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### CORRECTIONS

On page 693, Vol. 99, No. 3, February, 1933, change the paragraph beginning on line 24 to read, "However, among the series of substances which were recently encountered in the determination of the position and size of Ring III of strophanthidin<sup>3</sup> was a *hydroxydihydrostrophanthidinic acid* (Formula V). This substance was formed by the addition of two hydroxyl groups to the double bond of monoanhydrodihydrostrophanthidin *and simultaneous oxidation of the aldehyde group to carboxyl* on oxidation with permanganate. *As the methyl ester it yielded in turn* . . ."

These substances and their interrelationships were correctly presented in the paper in which they were originally described (Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **97**, 727 (1932)).

On pages 694 and 695, Formula V, read *COOH* for the aldehyde group, *CHO*; Formulas VI and VII, read *COOCH<sub>3</sub>* for *CHO*.

On page 695, line 1 of the text, read *hydroxydihydrostrophanthidinic acid* for *hydroxydihydrostrophanthidin*.



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## IS THERE AN UNKNOWN COMPOUND OF THE NATURE OF CALCIUM CITRATE PRESENT IN THE BLOOD?

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The hypothesis that a considerable fraction of the diffusible calcium of the blood is present as a complex, negatively charged ion, in combination with some unknown organic substance, has become strongly entrenched in the literature. A large amount of experimental data has been accumulated purporting to support the existence of such a calcium compound. It is further assumed, in most instances, that this organic citrate-like compound is elaborated through the agency of the parathyroid hormone. An earlier view-point was that the substance might be the parathyroid hormone itself, but since it has been shown by Collip (1) and others that the parathyroid hormone is of colloidal magnitude, and is itself non-diffusible, the hypothetical calcium compound cannot be the parathyroid hormone. The finding of Hastings, Murray, and Sendroy (2) that parathyroid extracts have little influence on the solubility of the calcium salts of blood serum is also against this.

The existence of such a hypothetical citrate-like calcium compound has been proposed and supported most prominently by Greenwald (3), Sendroy and Hastings (4), Bernhard and Beaver (5), Klinke (6), von Beznák (7), and Brull (8). The experimental evidence which has been put forward by these investigators in support of the existence of this compound will now be considered and critically examined. Taken at their face value, the experiments offered make an imposing brief in favor of the theory. However, in our estimation, a great many of the experiments offered in favor of the existence of a complex calcium compound in the blood are either faulty in technique or when closely examined

do not at all bear the interpretation placed upon them. These shortcomings will be pointed out in the text.

Greenwald (3), in order to correlate what seemed to him the more significant observations on the tetany of parathyroidectomized animals, proposed the hypothesis that some of the serum calcium is bound to an organic citrate-like compound which has the power of maintaining calcium in solution in blood plasma in excess of the amount that normally would be soluble. He suggested that the substance might be the parathyroid hormone itself, or some substance elaborated through the agency of the parathyroid hormone. He offered no experiments of his own designed directly to test his hypothesis.

Hastings, Murray, and Sendroy (2) and Sendroy and Hastings (4) from their study of the solubility of the slightly soluble bone salts,  $\text{CaCO}_3$  and  $\text{Ca}_3(\text{PO}_4)_2$ , concluded that in the plasma the calcium is normally not in a state of supersaturation with respect to these salts, but that the calcium exists in an abnormal amount bound to some substance besides the proteins, which holds it in solution in an unionized form.

These authors determined the stoichiometric solubility product of  $\text{CaCO}_3$  and  $\text{Ca}_3(\text{PO}_4)_2$  in mixed sodium chloride-sodium bicarbonate solutions, with added amounts of calcium and phosphate when desired, as a function of the ionic strength of the solution. The value obtained in this way at  $\mu = 0.160$ , the calculated ionic strength of blood serum, they assumed would be the solubility product of these salts in blood, provided that the blood serum behaved as a simple electrolyte solution. On shaking serum with  $\text{CaCO}_3$ , they found the resulting ion product,  $[\text{Ca}^{++}][\text{CO}_3^{--}]$ , was higher than that obtained with the simple salt solutions. In their opinion, this effect was explainable by the influence of the protein and an unknown, slightly ionized compound,  $\text{CaX}$ , being present in blood serum. Largely, the validity of this opinion centers around the point of whether a solubility equilibrium was attained in the serum saturation experiments. It seems reasonable to hesitate about accepting the claim that such an equilibrium was established when it is considered that these investigators found serum shaken with solid  $\text{CaCO}_3$  showed no change in calcium concentration even when the calcium of the original serum was considerably augmented or depleted, as long as inorganic phosphate

was present in the solution. Besides this, it is also uncertain that the solubility product obtained at the same ionic strength with the simple  $\text{NaCl-NaHCO}_3$  solutions will hold for the more complex mixture of ions present in blood serum.

When blood serum was shaken with  $\text{Ca}_3(\text{PO}_4)_2$  alone or with a mixture of  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{CaCO}_3$ , there was obtained a marked decrease in the calcium content. This would seem to indicate that serum at least was supersaturated with respect to  $\text{Ca}_3(\text{PO}_4)_2$ , if not with respect to  $\text{CaCO}_3$ . However, it was found that not  $\text{Ca}_3(\text{PO}_4)_2$ , but  $\text{CaCO}_3$  instead was precipitated in these experiments, a finding which has been confirmed by Klinke (6). On this ground, Sendroy and Hastings reject even the view that there is a supersaturation with respect to  $\text{Ca}_3(\text{PO}_4)_2$ . They offer no explanation for the drop of the calcium in this way on shaking serum with  $\text{Ca}_3(\text{PO}_4)_2$ , but Klinke believes it is due to the adsorption of a negatively charged complex ion by the positively charged  $\text{Ca}_3(\text{PO}_4)_2$ .

These few paragraphs are sufficient to show that no strongly positive conclusion for the existence of an unknown, slightly ionized calcium compound can be drawn from the work of Hastings, Murray, and Sendroy and Sendroy and Hastings.

From results obtained by electrodialyzing serum through collodion and parchment membranes, Bernhard and Beaver (5) concluded that a portion of the blood calcium is present in the form of a negatively charged complex ion, perhaps through a combination in some unknown way with the plasma phosphate and possibly also the magnesium. These views are based on the results that in seven out of their twenty-six electrodialysis experiments they were able to show the presence of calcium in the anode compartment and in five of these experiments magnesium also was detected in the anode. The authors also state that in some instances inorganic phosphate was found in the cathode compartment. Why no calcium migrated to the anode in the nineteen other experiments is not explained. Furthermore, even in the positive cases, there was no constancy in the results, the amount of calcium found in the anode varying from a mere trace to values greater than the calcium migrating to the cathode compartment. Because of the inconstancy and the variability of Bernhard and Beaver's experimental results, and in view of the results of our own electrodialysis experiments, we believe that the few so called posi-

tive results obtained by these authors may have resulted from the use of faulty membranes.

A technical defect that is to be noted in Bernhard and Beaver's experiments is that the anode compartment was not prevented from becoming strongly acid. If the hypothetical calcium compound is similar in properties to the combination formed with citrate, then in acid solution it would decompose to give calcium ion and the unionized acid. With this condition prevailing, the calcium would tend to migrate back again toward the cathode, thus greatly decreasing the chance of detecting the wandering of calcium toward the anode if it does take place.

Among the champions of the theory that part of the blood calcium is combined with some unknown organic compound which exists in the blood as a negatively charged complex ion, Klinke (6) has been one of the ablest. He rejects the view that blood serum is a solution which is supersaturated with respect to  $\text{Ca}^{++}$ ,  $\text{PO}_4^{=}$ , and  $\text{CO}_3^{=}$  and instead asserts that the experimental observations are better in agreement with the hypothesis of the existence of a complex soluble calcium compound. In support of this Klinke offers the experimental results obtained by himself and others on the measurement of the calcium ion activity in serum, the difference in behavior between serum ultrafiltrates and artificial inorganic serum solutions on freezing, the results of electrical transference experiments, and finally the evidence from shaking experiments on serum and serum ultrafiltrates with use as the solid phase of  $\text{BaSO}_4$ ,  $\text{Ca}_3(\text{PO}_4)_2$ , and other adsorbents.

The value of the calcium ion activity of blood serum has been reported to be about 2.0 mg. per cent by a number of authors, who used various experimental procedures, none of which is above reproach. This value is offered in favor of the calcium complex ion theory. Klinke thinks that the results obtained by Corten (9) in this field are particularly trustworthy and carry great weight.

Corten determined the calcium ion activity by the use of the electrode,  $\text{Zn} | \text{ZnC}_2\text{O}_4, \text{CaC}_2\text{O}_4 | \text{Ca}^{++}$ . This electrode is claimed to be theoretically objectionless and by its use an average value of about 2.0 mg. per cent of calcium was found to be ionized in blood serum, in agreement with the figures reported by other workers. However, this electrode for calcium ion, when tried in our laboratory, gave erratic results even with solutions of simple inorganic calcium salts so that practically it is far from being objectionless.

It is stated by Klinke that when serum ultrafiltrates are frozen, no precipitation of calcium salts occurs, but when artificial inorganic serum solutions are frozen, a precipitation of calcium takes place. This argues against a state of supersaturation in the serum. While we have no direct evidence to offer on this experiment, except to suggest that not the state of saturation but the presence of the organic compounds in blood acting as protective substances may account for the difference in behavior, it is worth while here to mention the results of certain analogous experiments which we carried out. In these experiments, serum ultrafiltrates were evaporated at about 50° by means of a blast of air almost to dryness. On extracting the residues with ethyl alcohol, acetone, and even water, no calcium was found in the extracts, showing the calcium to have been completely precipitated during the evaporation. A citrate-like calcium compound, were it present, unless it becomes decomposed during the drying, might be expected to be redissolved by one or more of the extracting reagents.

Among his results which Klinke feels very strongly support the existence of a negatively charged complex calcium ion in the blood, are those obtained from a comparison of the electrical transference of serum ultrafiltrates and artificial serum solutions. With serum ultrafiltrates, he found that in an electrical field the calcium of the ultrafiltrate migrated to an almost equal extent to both the anode and cathode compartments, whereas in a similar experiment with an artificial serum solution, the calcium migrated preponderately to the cathode as theoretically it all should. Yet it is to be noted that Klinke's experiments on this point are by no means completely convincing. The number of runs cited are only two for the ultrafiltrates and one for the inorganic serum solutions. Reversible electrodes were not used, so there was plenty of opportunity for complicating physical and chemical changes and lastly, even in the experiment with the inorganic serum solution, 20 per cent of the calcium present was found in the anode compartment. Our own results on the electrical migration of calcium in serum ultrafiltrates do not harmonize with those of Klinke.

The views of Klinke have found a strong supporter in von Beznák (7).<sup>1</sup> Klinke and von Beznák maintain that the existence

<sup>1</sup> Although it is somewhat foreign to the topic under consideration, it seems desirable to comment on what von Beznák terms the "physiologically diffusible calcium" of the blood. This is defined as the quantity of calcium

of a complex calcium compound in blood is demonstrated by the fact that on shaking serum with such solids as  $\text{Ca}_3(\text{PO}_4)_2$ , bone,  $\text{BaSO}_4$ , and  $\text{MgNH}_4\text{PO}_4$  (positive adsorbents) calcium is removed, whereas the calcium remains unchanged when serum is shaken with kaolin or animal charcoal (negative adsorbents) (see Klinke (6)). On the serum of normal dogs, von Beznák found an average decrease of about 40 per cent of the total calcium when shaken with powdered bone and about a 50 per cent decrease when shaken with  $\text{BaSO}_4$ . The serum of parathyroprivic dogs, when shaken with the bone, showed a much smaller decrease in calcium; indeed, in some instances, there was even an increase. This action of bone and  $\text{BaSO}_4$ , it is assumed, represents an adsorption and since the amount removed from parathyroid-deficient serum is less, it indicates a decreased formation of the negatively charged calcium complex. Bone from parathyroidectomized dogs was found to have identically the same properties in this respect as bone from normal animals.

Aside from the difference in action between the positively and negatively charged solid, no further proof is offered that the removal of calcium is due to an adsorption. For instance, it is not shown that the state of subdivision or the quantity of the solids has any effect, as might be expected for an adsorption. In the shaking experiments of von Beznák, only the changes

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which can pass through the capillary walls into the tissues. According to von Beznák, this quantity cannot be determined by the use of any artificial membrane, but only by the amount of calcium which is present in the extracellular fluids which can be considered to be ultrafiltrates of the blood plasma. Von Beznák considers the lymph to be such a fluid and since lymph contains between 80 and 90 per cent of the amount of calcium found in serum, this amount in his mind represents the "physiologically diffusible calcium" of the blood. In this formulation, von Beznák neglects the important point that the definition of diffusibility, even in this instance, implies that the capillary walls are impermeable to the colloids of the blood and accordingly, if the calcium content of any extracellular fluid is to be used as a measure of this quantity, it must be a fluid which is protein-free. Lymph certainly does not fulfil this requirement, since it contains between 3 and 4 per cent of protein. The various nearly protein-free transudates and exudates which have been analyzed give values for calcium which are much lower than for lymph and are in substantial agreement with the results obtained by ultrafiltration or dialysis through collodion membranes.

undergone by the calcium of the serum are recorded, and nothing whatsoever is stated about the inorganic phosphate, the carbon dioxide, and the pH. Yet it takes but little reflection to see that these variables, besides the state of the calcium, might be expected to contribute toward the results obtained.

In attempting to test the views of von Beznák that the loss of calcium on shaking serum with bone is due to the adsorption of a calcium complex, we carried out a few shaking experiments using bone and  $\text{CaHPO}_4$  as the solid phase on inorganic serum solutions. Our basis of reasoning in these experiments was, if the adsorption idea is correct, a different effect might be expected in the shaking experiments with inorganic serum solutions than with blood serum, since the inorganic solutions do not contain the negatively charged calcium complex assumed to exist in blood.

The experiments were carried out in a thermostatically controlled oil bath at the temperature of  $25^\circ$ , in flasks fitted with siphon tubes, so arranged that samples of the liquid could be removed at any time during the experimental period without any loss of  $\text{CO}_2$  from the gas phase. The inorganic serum solutions were prepared from a stock solution containing 1.70 gm. of  $\text{NaHCO}_3$  and 6.0 gm. of  $\text{NaCl}$  per liter to which the desired amounts of calcium chloride and sodium phosphate were added as required. The powdered bone used, either rat or beef, was prepared by removing as much of the marrow and organic matter as was possible and then pulverizing and drying the bone. The analyses were carried out with the Kramer-Tisdall method for calcium and the Fiske-Subbarow method for phosphate. Powdered bone and  $\text{CaHPO}_4$  were chosen for use because bone was the most extensively used material in the experiments of von Beznák and  $\text{CaHPO}_4$  because, according to Shear, Washburn, and Kramer (10) and Shear and Kramer (11), inorganic serum solutions and blood serum reach a true and rapid saturation equilibrium when it is present as the solid phase. By a comparison of the action of these two solids, it was expected to gain further information on the mechanism of the shaking experiments.

In Table I are tabulated the results. This table is constructed so as to facilitate comparison with the results of von Beznák (7) and the time of shaking shown is about the maximum used by him. An examination of Table I shows that with both bone and  $\text{CaHPO}_4$

there is qualitatively the same picture as found by von Beznák on serum.  $\text{CaHPO}_4$ , it is true, removes considerably less calcium than bone in the same period of shaking. When the data for

TABLE I  
*Effect of Powdered Bone and  $\text{CaHPO}_4$  on Calcium Distribution of Organic and Inorganic Serum Solutions with Time of Shaking*

Sample	Shaking time	Solid	Ca before shaking	Ca after shaking	Difference	Remarks
	hrs.		mg. per 100 cc.	mg. per 100 cc.	per cent	
Rabbit Serum 1	7.5	Bone	17.8	11.8	-34	
" " 2	5	"	15.8	12.7	-20	
Serum, Dog L.	5	"	8.6	3.4	-62	
" " P	5	"	11.8	5.0	-58	
" " B	3	$\text{CaHPO}_4$	11.2	7.2	-36	
" " "	3	"	14.7	10.9	-26	Injected with 100 units parathormone
" " W	6	"	10.8	9.0	-17	
Rabbit Serum 2	5	"	15.8	13.4	-15	
Inorganic serum solutions						
Solution A	5	Bone	9.4	2.1	-55	Contained no phosphate originally
" "	5	$\text{CaHPO}_4$	9.4	5.7	-39	" "
" B	4.5	Bone	10.4	8.2	-21	" "
" C	5	"	7.7	5.8	-25	" "
" "	5	$\text{CaHPO}_4$	7.7	6.4	-17	
" D	2	Bone	7.7	6.4	-17	Phosphate, 4.0 mg. P per 100 cc.
" "	2	$\text{CaHPO}_4$	7.7	4.9	-36	
" E	4.5	Bone	10.5	7.3	-30	Phosphate, 4.5 mg. P per 100 cc.
" F	5	"	7.8	5.4	-31	Phosphate, 4.0 mg. P per 100 cc.
" "	5	$\text{CaHPO}_4$	7.8	6.5	-17	
" G	5	Bone	0	1.3		No phosphate of calcium originally present
" "	6	$\text{CaHPO}_4$	0	3.4		

inorganic serum solutions are examined, it is observed that when the amounts of calcium present were of the order found in blood serum, the shaking experiments show the same picture as found with blood serum. On the other hand, with none or very little

calcium to start with, there was an increase in the calcium content as might reasonably be expected on the basis of a solubility effect. The agreement between the picture for blood serum and for inorganic solutions with comparable amounts of calcium, it seems to us, does not favor the theory of Klinke and of von Beznák.

The results obtained in the shaking experiments by von Beznák when sodium citrate was added to the blood serum, are also of interest in connection with the adsorption question. In these experiments, there was a large increase in the calcium in solution. To von Beznák this indicated that the parathyroid hormone may function in a similar manner by dissolving the bone salts to bring calcium into solution in the body fluids and the blood in the form of the negatively charged complex calcium ion postulated by this author and others. Apparently, it did not occur to von Beznák that the results obtained with citrated serum appear to be contrary to the view-point that the action of bone powder is to adsorb a negatively charged calcium-containing complex. Since citrate forms with calcium the prototype of just that kind of a complex which is presumed to be adsorbed from the blood by bone powder, it is strange that instead of the bone powder removing a greater amount of calcium from citrated serum, there is actually an increase of the calcium in solution.

It may well be asked at this point, if, aside from the strength or weakness of the evidence so far cited in favor of the complex organic compound of calcium in blood, the recent demonstration that citric acid is a normal constituent of the blood is not a verification of these theories and that the complex may well be a combination of calcium citrate. Indeed, Brull (8) takes the view-point that such is the case, basing his view on the results of a study of the excretion of calcium by the isolated dog kidney. He found that injection of phosphate decreased the excretion of calcium, whereas, after the injection of citrate, there was a large increase in the urinary output of calcium. This led him to the view that such a citrate-like compound is normally present in the blood and that only calcium in this form is capable of being excreted by the kidney, whereas it is totally unable to excrete ordinary calcium cations from the blood. As further evidence for his views, Brull cites the work of Thunberg (12), who found between 250 and 1800 mg. of citrate (calculated as citric acid) per liter of urine and ac-

cordingly proposes that a calcium citrate may be the compound in question.

From the urine figures he estimates that there ought to be between 5 and 30 mg. of citrate present per 100 cc. of blood. However, more recent work does not confirm the reasoning of Brull. Fasold (13) has shown that citrate is not an obligatory urinary constituent and its presence is mainly determined by the diet; the quantity that is excreted being largely dependent on the amount and kind of vegetables ingested. Also, the latest work on the citrate content of blood by Schersten (14) shows the amount present to be highly variable and to range between the figures of 1 to 4 mg. of citric acid per 100 cc. of serum. From these figures it would seem that a complex calcium citrate can only account for a small fraction of the amount of the organic complex calcium required by the theory that such a compound determines the height of the calcium in blood. Citric acid has a formula weight of 192 and since it could hardly hold more than 1 mol of calcium in complex combination per mol of citric acid, the citrate in the blood could in the most extreme case only account for less than 1 mg. of calcium per 100 cc. of calcium in solution. Moreover, the requirement of the other factor of the theory, namely that the amount of complex is determined by the parathyroid hormone, is not well met by citrate, since no evidence has been offered whatsoever that the citrate content of the blood or its urinary excretion is in any way determined by parathyroid activity.

Besides the criticism directed against the experimental evidence cited in the survey above, and the work already mentioned, we also wish to offer certain experimental results on the electrodialysis of blood serum and serum ultrafiltrates.

*Electrodialysis Experiments*—Electrodialysis was carried out in a three compartment glass cell, each of the compartments having a capacity of about 60 cc. This cell was constructed by cutting pieces of the proper size from 5 cm. diameter heavy walled Pyrex glass tubing, blowing a hole through the surface of each section to serve as a means of filling and draining the compartment, and then grinding the ends of each section to a flat surface so as to make a tight junction. Rubber stoppers were fitted into the end sections and the whole cell was held together by means of a suitably constructed clamp. Cellophane was used for the membrane and the

electrodes were of platinum. In making a run, the serum or the ultrafiltrate used for the experiment was introduced into the middle compartment, the cathode compartment was filled with distilled water, and the anode with a solution of 0.35 N  $\text{NH}_4\text{OH}$ . The ammonia served as a weak base to maintain the anode compartment at an alkaline reaction during the dialysis and so prevent the possible dissociation of the calcium complex—assuming it to be present—by the developing acidity, in the manner already discussed. That the anode compartment remained alkaline was tested for from time to time by pipetting out a few drops into a test-tube containing alizarin red. When it showed signs of becoming acid, the liquid of both anode and cathode compartments was drained into labeled flasks and fresh portions of ammonia and distilled water were added respectively to the electrode compartments. Usually, during the course of a run, three changes of the compartment liquid were required. The electrodialysis was carried on from a 110 voltage line with a current strength of about 0.5 amperes until the drop in current showed that most of the electrolyte had migrated from the middle into the electrode compartments.

In analyzing for calcium and phosphate ion, each of the three compartment portions were made up to definite volume and aliquots used for the analysis. Where serum was used in an experiment, the protein of the serum from the middle compartment was precipitated by adding an equal volume of 10 per cent trichloroacetic acid. To determine calcium, aliquots were pipetted into conically pointed centrifuge tubes of 50 cc. capacity, the acidity was adjusted to the turning point of brom-cresol green, saturated ammonium oxalate was added, and the rest of the procedure carried out after the manner of the Kramer-Tisdall (15) method. Inorganic phosphate was determined by the Fiske-Subbarow (16) method.

The results of the electrodialysis experiments are recorded in Tables II and III. Table II gives the figures obtained on blood sera and Table III the results with serum ultrafiltrates. To make the conditions for the detection of the hypothetical complex calcium ion as favorable as possible, rabbit blood was selected for use, because the calcium content of rabbit serum is extraordinarily high and a large fraction of the calcium is diffusible. If a complex

calcium compound is responsible for the stability and height of the diffusible calcium, then rabbit serum should contain normally a much higher content of this compound than the sera of most

TABLE II  
*Electrodialysis Experiments on Serum*

Experiment No	Serum used	Time of run	Serum		Calcium distribution at end			Phosphate distribution at end		
			Ca per 100 cc	P per 100 cc	Middle	Cathode	Anode	Middle	Cathode	Anode
		hrs.	mg	mg.	mg	mg	mg.	mg	mg	mg
2	50 cc Serum 1	4 3	14 2	10 8	3 00	3 00	0 0	1 05	0 0	4 65
12	50 " " 2	3 0	13 1	9 0	2 20	3 80	0 0	0 50	0 0	3 90
15	50 " " 3	3 5	13 2	10 7	2 40	3 45	0 0	2 05	0 0	3 60
20	50 " " 3	2 75	13 2	10 7	3 20	2 35	0 0	2 10	0 0	3 30
5	50 cc. Serum 1 + 0.1 gm. Na citrate	2 5	14 2	10 8	2 70	2 60	0 50	1 60	0 0	4 40
6	50 cc. Serum 2 + 0.5 gm. Na citrate	2 5	13 1	9 0	4 05	1 15	1 70	1 40	0 0	2 90
13	50 cc Serum 2 + 0.7 gm Na citrate	2 0	13 1	9 0	4 6	0 45	1 40	2 25	0 0	2 60
14	35 cc. Serum 2 + 0 35 gm. Na citrate	2 0	13 1	9 0	0 60	1 00	0 50	0 0	0 0	1 90
22	50 cc Serum 3 + 0 10 gm Na citrate	3 75	13 2	10 7	3 10	3 20	0 05	1 65	0 0	3 85
23	50 cc. Serum 3 + 1 0 gm. Na citrate	4 75	13 2	10 7	3 85	1 50	0 70	0 85	0 0	4 80
24	45 cc. Dog 1*	3 25	20 0	4 6	3 15	4 50	0 0	0 85	0 0	0 95
25	25 " " 1*	1 75	20 0	4 6	2 5	3 30	0 0	0 45	0 0	1 10

\* Serum from a dog injected with 200 units of parathormone.

other animals. With only a single exception, as the tables of data show, in unaltered serum or serum ultrafiltrate, no migration of calcium was detectable. No importance at all is attached to the

single exception, it probably being due to the use of a faulty membrane. Our negative results, with the much more favorable conditions employed, it would seem, completely dispose of the claims of Bernhard and Beaver that calcium migrates to the anode in such experiments. As another extreme test of this, there are listed in Experiments 24 and 25 the electrodialysis results obtained

TABLE III  
*Electrodialysis Experiments on Serum Ultrafiltrates*

Experiment No.	Ultrafiltrate used	Time	Ultrafiltrate		Calcium distribution			Phosphate distribution		
			Ca per 100 cc.	P per 100 cc.	Middle	Cathode	Anode	Middle	Cathode	Anode
		hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
3	50 cc. Serum 1	2.5	7.9	10.8	1.20	0.75	0.0	0.65	0.0	2.8
4	50 " " 1	4.5	7.9	10.8	0.25	2.60	0.0	0.15	0.0	2.30
8	50 " " 2	3.0	8.0	8.8	0.95	0.60	0.0	0.50	0.0	2.50
9	50 " " 2	2.0	8.0	8.8	2.80	0.75	0.15	1.50	0.0	2.20
11	50 " " 2	2.5	8.0	8.8	1.15	1.00	0.0	0.90	0.0	2.30
16	43 " " 3	3.25	7.2	10.0	1.95	1.20	0.0	0.75	0.0	4.60
7	50 cc. Serum 2 + 0.22 gm. Na citrate	2.5	8.0	8.8	0.65	1.20	0.70	0.0	0.0	3.35
10	50 cc. Serum 2 + 0.4 gm. Na citrate	3.0	8.0	8.8	1.00	1.70	0.90	0.50	0.0	3.30
18	50 cc. Serum 3 + 0.05 gm. Na citrate	3.5	7.2	10.0	2.30	1.45	0.0	1.30	0.0	3.95
21	45 cc. Serum 3 + 0.5 gm. Na citrate	3.25	7.2	10.0	0.75	1.90	0.70	0.30	0.0	4.10

on the serum of a dog, in which the calcium value had been raised to the extremely high value of 20 mg. per 100 cc. by parathormone injection. Here it would seem, if the theory is correct that the parathyroid hormone acts by mobilizing an organic substance capable of forming a negatively charged complex ion with calcium, that there should have been a migration of calcium to the anode. On the contrary, no trace of such a migration was detectable.

To get an indication of the behavior that might be expected with a negatively charged calcium ion complex, experiments were carried out on serum and serum ultrafiltrates to which varying amounts of citrate were added. The results obtained are shown in the appropriately indicated sections of Tables II and III. These experiments show that with the definite presence of a negatively charged calcium ion in sufficient amounts, there does take place a migration of calcium to the anode compartment and roughly speaking, the larger the amount of citrate present, the greater is the amount of calcium migrating to the anode. However, these experiments with added citrate also point to the possibility that electrodialysis may not be a sufficiently delicate means to test the presence of the postulated calcium complex in normal blood in the amounts presumed to exist there, since citrate to the extent of about 100 mg. of citric acid per 100 cc. of serum was required to get a detectable amount of anode migration.

Our experiments also are negative on the possibility of phosphate ion being involved, since its migration as shown in Tables II and III is quite independent of the calcium.

This exposition, we feel, shows that there is almost no positive evidence for the presence of such an organic negatively charged calcium ion complex in the blood as has been postulated. On the other hand, none of the evidence we have offered completely rules out the possible existence of such a substance, nor do we wish to imply that we categorically deny that such a calcium compound may exist.

It still remains for future work of a more decisive nature to settle this question.

#### SUMMARY

It has been shown that there is no strongly positive evidence for the existence of an organic citrate-like compound of calcium in the blood.

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# THE DETERMINATION OF MAGNESIUM IN BLOOD WITH 8-HYDROXYQUINOLINE

A NOTE ON THE PAPER BY GREENBERG AND MACKEY

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In the May, 1932, number of this *Journal*, Greenberg and Mackey<sup>1</sup> presented a paper in which they described a method for the determination of magnesium in blood, serum, and plasma with 8-hydroxyquinoline, which depends upon the bromination of the quinoline in the magnesium-8-hydroxyquinoline precipitate. This method had already been published by me in October, 1931<sup>2</sup>. In referring to my method the authors say: "When our experimental work was about completed, there appeared a paper by Bomskov, for the determination of magnesium in serum by hydroxyquinoline, which also employs the bromination reaction. In other respects, our method differs largely from and, we feel, is superior in point of simplicity to both those of Bomskov. . . ." These points of difference are (1) only one precipitation is used because of the calcium having been removed by working with oxalated blood; (2) the precipitate of magnesium hydroxyquinoline is isolated by filtration instead of centrifugation; (3) for the bromination of hydroxyquinoline a considerable excess of bromine is used.

I cannot feel that my method differs largely from that published by Greenberg and Mackey. As regards simplicity, only the first point is an improvement. However, in the clinical laboratory it is of the greatest interest to determine the calcium and the magnesium in the same blood sample. This can be done in the trichloroacetic acid filtrate which I use for the magnesium determination, but not in the oxalated blood or plasma which is used by Greenberg and

<sup>1</sup> Greenberg, D. M., and Mackey, M. A., *J. Biol. Chem.*, **96**, 419 (1932).

<sup>2</sup> Bomskov, C., *Z. physiol. Chem.*, **202**, 32 (1931).

Mackey. The second point, the isolation of the precipitate by filtration instead of centrifugation, cannot be deemed a step in the direction of simplicity. I myself think it quite simple to precipitate, to wash, to centrifuge, and to titrate in the same centrifuging tube, without filtering the precipitate. The third point is a theoretical one. In my magnesium determinations, I used an excess of bromine equal to twice the theoretical amount for the bromination of the hydroxyquinoline. I found this to be a sufficient amount to give rapid bromination. Greenberg and Mackey increase this excess of bromine considerably but I do not feel that this is a point of great importance.

In our laboratories we have determined the magnesium content of about 200 blood samples. The following is a résumé of some of our results. The magnesium content of the blood serum of six normal men, varying in age from 58 to 62 years, ranged from 1.7 to 2.6 mg. per cent; that of normal children, varying in age from 4 months to 13 years, ranged from 1.3 to 2.5 mg. per cent; that of children with florid rickets, varying in age from  $1\frac{1}{4}$  to 10 months, ranged from 0.8 to 1.1 mg. per cent.

## THE DETERMINATION OF MAGNESIUM IN BLOOD WITH 8-HYDROXYQUINOLINE

A REPLY

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(Received for publication, July 18, 1932)

In answer to Dr. Bomskov's communication<sup>1</sup> we would make the general reply that the final judgment of the merits of an analytical method rests in the hands of the laboratory workers who use it rather than on arguments advanced on paper and we are willing to leave the decision of the relative merits of our procedure for magnesium as against Bomskov's with them. Indeed such a point as to whether filtration or centrifugation is superior to use in isolating a precipitate can only be settled by test and not by opinion. Our work favors the micro filtration method.

Dr. Bomskov concedes the advantage of only a single precipitation, but sets against this the claim that both calcium and magnesium can be determined on the same trichloroacetic acid filtrate of blood serum according to his procedure. This is rather curious, since no mention of such a thing is made in his original paper, whereas in our communication there is given an outline for this very purpose. Nor is the use of oxalated blood for magnesium analysis to be deprecated, since it offers a simple means of determining the magnesium of the corpuscles, the study of which has, up to now, been neglected.

The results of studies on the plasma and corpuscle magnesium in health and in various pathological conditions carried out by our method, will shortly be submitted for publication.

<sup>1</sup>Bomskov, C., *J. Biol. Chem.*, **99**, 17 (1932-33).



## COMPOSITION OF BONE

### XIII. DIRECT GRAVIMETRIC DETERMINATION OF Ca, Mg, AND PO<sub>4</sub>\*

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#### INTRODUCTION

It was pointed out by Shear and Kramer (1928) that the literature contains few analyses of bone accurate enough to serve for the determination of the molecular constitution of the calcium phosphate present. Some analysts reported an excess of calcium over the amount required to fit the formula  $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaCO}_3$ ; others reported an excess of phosphate. These deviations from the theoretical composition have usually been ascribed either to shortcomings of the analytical methods employed or to experimental error. It is not impossible, however, that these discrepancies may have been due to actual variations in the composition of bone, and not to faulty analytical technique.

A similar situation was noted as regards  $\text{Ca}_3(\text{PO}_4)_2$  prepared by precipitation from solution. Precipitates, presumably of the composition and constitution indicated by this formula, rarely gave analytical results that fitted the formula. Here, too, the discrepancies have usually been attributed to errors of analysis; but here, too, the possibility exists that the differences may have been due to variations in composition and not to analytical errors.

It is therefore desirable to obtain analytical data on bones, and on precipitates of calcium phosphate, by methods capable of differentiating between these possibilities. The standard macroanalytical procedures are not suitable for this purpose. Although the determination of Ca, of Mg, or of PO<sub>4</sub> may be made

\* A preliminary report of this study was published in *Proc. Soc. Exp. Biol. and Med.*, **29**, 625 (1932).

with a high degree of accuracy in a pure solution which contains only one of these ions, the methods are less accurate when applied to mixtures. It is a comparatively easy matter to get results accurate to 1 or 2 per cent in mixtures; the difficulties arise when more accurate results are desired. The numerous studies and conflicting recommendations reported in the literature attest to the difficulties presented by this analytical problem.

#### LITERATURE

*Calcium*—The separation of Ca from Mg as the oxalate has not proved entirely satisfactory. Some Mg is usually found in the Ca precipitate; some of the Ca frequently remains unprecipitated and contaminates the Mg precipitate, as was pointed out, for example, by Hillebrand and Lundell (1929). Reprecipitation of the Ca minimizes one error but increases the amount of the other error.

The proper pH for the calcium oxalate precipitation is also a moot question. Many analysts make the solution alkaline. Dobbins and Mebane (1930) obtained good results for Ca in the presence of  $\text{PO}_4$ , but this method cannot be employed when Mg is also present, for reasons pointed out by Shohl (1922). As Popoff, Waldbauer, and McCann (1932) recently stated, "The quantitative separation of calcium and magnesium has always been a time-consuming and rather uncertain procedure. The large number of papers pertaining to the separation of calcium as the oxalate, and the equally large number of opinions expressed are ample proof that the problem deserves further study."

There is also wide difference of opinion as to how the calcium precipitate is best weighed. Ignition to CaO is frequently used. Winkler (1918), Luff (1925), and Szebellédy (1927) weighed the precipitate as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ . Foote and Bradley (1926) and Willard and Boldyreff (1930) recommended conversion of the calcium oxalate to  $\text{CaCO}_3$  for, they stated, "Weighing as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$  always involves the danger of a slight variation in the water content; weighing as  $\text{CaC}_2\text{O}_4$  is objectionable because of its hygroscopicity, and the errors in weighing CaO are too well known to need mentioning."

*Phosphate and Magnesium*—Epperson (1928) stated, "Probably no determination in analytical chemistry has been the subject of

a greater number of conflicting statements than that involving the precipitation of magnesium ammonium phosphate." Hillebrand and Lundell (1929) stated, "A great deal of uncertainty exists concerning the proper conditions for determining magnesium as phosphate in spite of the enormous amount of work that has been done."

The numerous studies since the classic contribution of Neubauer (1894, 1896) have led to diametrically opposed conclusions. Some investigators reported that precipitation at room temperature leads to erroneous results, while others concluded that only when precipitation is carried out at room temperature can correct results be obtained. Uncertainty also exists regarding the proper concentrations of the various reagents, the order and the rate of their addition, etc. The ignition of the precipitate has proved to be a source of trouble. Neubauer showed that considerable  $P_2O_5$  may be lost by volatilization. Since then the temperature and mode of ignition have received considerable attention, and conflicting procedures have been recommended; Hoffman and Lundell (1930) have recently made an important contribution to this aspect of the subject.

In a review of the work of others and of his own series of investigations, Balereu (1921, 1930) pointed out that the methods of the earlier analysts (Gibbs, Neubauer, Schmitz, Järvinen, Jørgensen), which have received wide acceptance, are all methods in which opposing errors compensate each other more or less. He concluded, "Es fehlt an einer Methode zur Mg-Bestimmung, die nicht Kompensationsmethode ist."

No attempt has been made here to review the voluminous literature on the analysis of calcium, magnesium, and phosphate; citations have been made from a few contributions only to show that many of the difficulties encountered by earlier analysts are still sources of error today.<sup>1</sup> If an accuracy of 1 or 2 per cent is desired, it is comparatively easy to devise a widely applicable scheme of analysis. But when more precise results are desired, each situation becomes a separate problem and requires separate consideration. Our aim was not to devise a general analytical

<sup>1</sup> The comments and discussion throughout this paper refer to *macro precision methods* and not to the widely used micro methods in which this high degree of accuracy is not required.

method, but rather a method for analyzing solutions containing Ca, Mg, and  $\text{PO}_4$  in the relative concentrations in which they are present in bone. The technique finally adopted gave results of the desired accuracy.

#### EXPERIMENTAL

*Standard Solutions*—Analyzed reagent  $\text{CaCO}_3$  was washed with water, dried at  $110^\circ$  to constant weight, dissolved in  $\text{HCl}$  or  $\text{HNO}_3$ , heated carefully to drive off  $\text{CO}_2$ , and made up to volume.

Analyzed reagent  $\text{KH}_2\text{PO}_4$  was recrystallized from water and was reprecipitated (*a*) from a saturated solution by alcohol at room temperature, according to the suggestion of Holcomb and McKibbin (1928), and (*b*) from a 0.2 M solution by alcohol at  $80^\circ$ . The precipitates were dried at  $37^\circ$ . The loss on ignition<sup>2</sup> was 13.2 per cent for (*a*) and 13.3 per cent for (*b*). A third preparation was dried at  $110^\circ$  after recrystallization from water.<sup>3</sup>

Analyzed reagent  $\text{MgCl}_2$  was recrystallized from water and dried at  $37^\circ$ . Solutions of this salt were evaporated in silica beakers with a slight excess of  $\text{HgO}$  and ignited to constant weight, according to the method of Fresenius (1907). Standard solutions were made from the recrystallized  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , from  $\text{MgO}$  prepared from it by means of  $\text{HgO}$ , and also from a specimen of analyzed reagent  $\text{MgO}$  after ignition to constant weight.

*Reagents*—Analyzed reagent  $\text{HCl}$  and ammonium hydroxide (which contained traces of calcium) were redistilled and kept in paraffined bottles.

Alcohol and ether were redistilled over alkali.

3 M  $\text{H}_3\text{PO}_4$  was prepared by dilution of a specimen of analyzed reagent phosphoric acid which was free from Ca and Mg.

0.5 M  $\text{MgCl}_2$  was made from the recrystallized  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ .

$(\text{NH}_4)_2\text{C}_2\text{O}_4$  and  $\text{H}_2\text{C}_2\text{O}_4$  solutions were made from recrystallized analyzed reagents.

<sup>2</sup> According to Murray (1927), "The loss on ignition should be not less than 13.13% nor more than 13.33 %."

<sup>3</sup> Although Olsen (1926) gives the melting point of  $\text{KH}_2\text{PO}_4$  as  $96^\circ$  we did not note any fusion at  $110^\circ$ . Clark (1928) recommends drying it at  $110$ – $115^\circ$ .

*Calcium*

*Direct Determination As  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$* —The general procedure employed in the determination of calcium was as follows: oxalic acid and  $\text{NH}_4\text{OH}$  were successively added to the Ca solution; after digestion on the steam bath, the precipitate was allowed to stand for periods ranging from 1 hour to overnight. The precipitate was filtered and washed, dried at about  $105^\circ$ , and weighed.

A number of conditions were varied: the precipitation temperature ranged between room temperature and  $90^\circ$ ; the excess of oxalate was varied from 25 to 275 per cent; the oxalate was added as ammonium oxalate or oxalic acid; the amount of Ca analyzed ranged from 40 to 200 mg.; the total volume was varied from 50 to 140 cc.; glass filter crucibles and Gooch crucibles were used; in some cases citrate was added to give a final concentration of from 6 to 30 mm; the pH was adjusted with NaOH as well as with  $\text{NH}_4\text{OH}$ ; the drying time was 1 hour and 48 hours; the pH was varied between 2.5 and 8.

The numerous variations are not described in detail, as most of them were found to be without important effect. The weights of the precipitates agreed, within a few tenths of a per cent, with the values calculated as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ . Some precipitates were dried for an additional 48 hours at  $105^\circ$  and then weighed again; the weights remained unchanged in all cases. The presence of 30 mm citrate did not affect the results; neither did adjustment of the pH with NaOH. However, low values were obtained on precipitation at room temperature.

These experiments showed that good results may be obtained for pure calcium solutions by direct weighing of the first precipitate as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ , provided the precipitation is carried out at about  $90^\circ$ . Furthermore, this compound is constant in composition and does not lose water on drying<sup>4</sup> at  $105^\circ$ . The average error of thirty-five analyses was  $\pm 0.2$  per cent.

*Comparison of Single and Double Precipitation*—Calcium was determined, in three different solutions, by both single and double

<sup>4</sup> The crucibles were not placed directly on the metal shelf of the oven, but on heat-insulating material such as asbestos or clay supports. Unless this precaution is taken the temperature of the precipitate is likely to be considerably above that registered by the thermometer suspended above the shelf.

precipitation; the analyses were made in triplicate. The pH was adjusted to the end-point of brom-cresol purple in all cases in both precipitations.

Fairly good results were obtained, in the absence of magnesium and phosphate, even by single precipitation. On the other hand, when Mg and  $\text{PO}_4$  were both present, high results were regularly obtained even by double precipitation.

*Single and Double Precipitation at Various Acidities*—It was thus obvious that the calcium precipitate was contaminated by the other constituents of the solution; the second precipitation reduced this contamination, but did not entirely eliminate it. A series of analyses was next performed to see what effect increased acidity would have on this contamination.

A solution containing 200 mg. of calcium, 6 mg. of magnesium, and 97 mg. of phosphorus was analyzed for calcium as described previously except that varying amounts of  $\text{NH}_4\text{OH}$  were added. Consequently, the calcium oxalate precipitates were obtained from solutions of different acidities; the pH range was 2.5 to 8.

Very high results were obtained in alkaline and neutral solutions, and high results were obtained down to a pH of about 6. As was found previously, reprecipitation reduced the amount of the contamination but did not entirely eliminate it; all the errors were plus errors (nine analyses). In each instance, reprecipitation was carried out at the same acidity as in the first precipitation.

Good results were obtained in the acid solutions (thirteen analyses), although we had expected to find values several per cent low in this region. Between pH 3 and pH 5, the second precipitates gave the same results as the first precipitates. Furthermore, the plus and minus errors of the second precipitates had about the same distribution, whereas above pH 6 the errors were all positive.

This unexpected finding led us to investigate further the precipitation of calcium oxalate in quite acid solution.

*Precipitation with Thymol Blue As Indicator*—With aliquot portions of a solution analogous to that just described, calcium was precipitated in the usual way, except that  $\text{NH}_4\text{OH}$  was added in the first pair of analyses to a pH of about 2, in the second pair to a pH of about 2.5, and in the last set of four analyses to a pH of about 3.

It was found that at pH values below 3 the precipitation of

calcium was incomplete; at about pH 3, however, all the calcium was precipitated as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ .

### *Phosphate*

*Determination As  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  in  $\text{KH}_2\text{PO}_4$  Solution*—Solutions which contained only  $\text{KH}_2\text{PO}_4$  were analyzed for phosphate by precipitation as magnesium ammonium phosphate. Omitting the variations, the general method employed was as follows: to the phosphate solution HCl and  $\text{MgCl}_2$  were added; then  $\text{NH}_4\text{OH}$  was added until blue to thymol blue; finally an excess of  $\text{NH}_4\text{OH}$  was added and the precipitate was allowed to stand overnight. An analogous procedure was followed in reprecipitation. All precipitations were carried out at room temperature. The precipitate was filtered by suction, washed with  $\text{NH}_4\text{OH}$ , with alcohol, and with ether; it was then dried at  $37^\circ$  for 1 hour, cooled, and weighed.

\* The amount of phosphorus analyzed varied from 13 to 48 mg., the excess of added Mg was varied from 150 to 550 per cent, the excess  $\text{NH}_4\text{OH}$  was varied from 0.2 to 0.5 M, and the total volume was varied between 125 and 290 cc.

The results obtained by the different variations of the general procedure are not described in detail because in general they were without important effect. Single precipitation usually gave high results and double precipitation usually gave satisfactory results. The average error of twenty-three analyses (double precipitation) was  $\pm 0.2$  per cent.

*Effect of  $\text{NH}_4\text{Cl}$* —In all cases enough HCl was added to the phosphate solution so that upon addition of  $\text{NH}_4\text{OH}$ , the final concentration of  $\text{NH}_4\text{Cl}$  was at least 0.3 M; the concentration of  $\text{NH}_4\text{Cl}$  was varied in these experiments between 0.3 M and 0.6 M. In general, variation of  $\text{NH}_4\text{Cl}$  between these limits did not affect the phosphate determination.

### *Magnesium*

*Determination As  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  in  $\text{MgCl}_2$  Solution*—The general procedure for the determination of magnesium was as follows: HCl was added to the magnesium solution so as to insure a final concentration of about 0.6 M  $\text{NH}_4\text{Cl}$ . After addition of  $\text{H}_3\text{PO}_4$ , concentrated  $\text{NH}_4\text{OH}$  was added. From this point on, the determination was essentially the same as that of phosphate.

The amounts of magnesium analyzed were 13.15 and 32.85 mg. The other variables were: total volume, 75 to 150 cc.;  $\text{PO}_4$  added, 12 to 40 times the Mg equivalent; excess  $\text{NH}_4\text{OH}$ , 0.3 to 0.5 M; and  $\text{NH}_4\text{Cl}$  present, 0.5 to 0.7 M. Within these limits, these variations did not significantly affect the value of the second precipitate. The average error of thirty-two analyses was  $\pm 0.2$  per cent.

When a 40-fold excess of phosphate was used, high results were regularly obtained; when ordinary alcohol, instead of alcohol redistilled over alkali, was used to wash the precipitate, almost half of the precipitate was lost. Citrate, in a final concentration of 6 to 30 mM, did not affect the Mg results, provided the excess of  $\text{NH}_4\text{OH}$  was sufficiently large (*cf.* p. 32).

*Stirring Time*—The effect of stirring time was also studied. In Epperson's "Standard procedure" the solution is stirred for about 5 minutes, and then 5 cc. excess of ammonia water are added, and the solution is again stirred for an additional 10 minutes. A series of twenty-seven gravimetric and volumetric analyses of Mg was performed, in which the stirring time was varied from 1 to 10 minutes after addition of all the reagents. Stirring for 1 minute gave as accurate results as stirring for 10 minutes.

#### *Analysis of Mixtures Containing Ca, Mg, and $\text{PO}_4$*

*Determination of Ca and  $\text{PO}_4$  in Presence of Ca, Mg, and  $\text{PO}_4$* —Analysts in the past have devised procedures for the analysis of Ca in the presence of Mg or of  $\text{PO}_4$ ; in a few instances procedures have been devised for the determination of Ca in the presence of both Mg and  $\text{PO}_4$ . There is, however, little information available on the determination of  $\text{PO}_4$  and of Mg, as well as of Ca, in such mixtures. By means of compensating errors, the determination of Ca may be accomplished in mixtures of Ca, Mg, and  $\text{PO}_4$  with results that differ from the correct Ca values by only a few tenths of a per cent; however, when Mg and  $\text{PO}_4$  are determined on the Ca filtrates, the results are often incorrect because of the errors which were inherited from the Ca determination. It is only in special cases that the errors which are made to compensate in the Ca analysis will be found to compensate also in the  $\text{PO}_4$  and Mg analyses of the Ca filtrate.

It has been shown above that single and double precipitation gave the same values for  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$  in pure Ca solutions, but

that when Mg and  $\text{PO}_4$  were also present, the value of the first Ca precipitate was too high. Reprecipitation gave better results for Ca, but subsequent analyses showed that when the first Ca precipitate is too high, low values are obtained for Mg and  $\text{PO}_4$ .

Fourteen determinations of Ca were made of solutions containing Ca, Mg, and  $\text{PO}_4$ , and in each instance  $\text{PO}_4$  was determined on the Ca filtrate. The aliquots analyzed contained from 40 to 100 mg. of Ca, from 19 to 48 mg. of P, and from 1 to 3 mg. of Mg. When Ca was precipitated at pH 6.3 the first Ca precipitate was about 1 per cent too large, and as a result the values for  $\text{PO}_4$  were about 1 per cent too low. At pH 3, correct results were obtained for Ca by single precipitation; the phosphate values were also correct.

Of all the analyses performed at pH 6.3, correct results were obtained for both Ca and  $\text{PO}_4$  in only two cases. In these two analyses the Ca precipitate was filtered off after standing for only 1 hour; under these conditions the errors just happened to compensate for both Ca and  $\text{PO}_4$ .

*Determination of Ca and Mg in Presence of Ca, Mg, and  $\text{PO}_4$ —*Thirty-six determinations of Ca were made of solutions containing Ca, Mg, and  $\text{PO}_4$ , and in each instance Mg was determined on the Ca filtrate. The aliquots analyzed contained from 100 to 400 mg. of Ca, from 48 to 194 mg. of P, and from 3 to 12 mg. of Mg. The pH was varied from 3 to 8. When the Ca was precipitated in alkaline, neutral, or slightly acid solution (pH 6.3), the Ca precipitate was too large by amounts that varied up to 2.5 per cent; in these cases the Mg precipitates were all too low, by amounts that varied up to 38 per cent.

On the other hand, when the Ca was precipitated in the neighborhood of pH 3, the Ca results were correct; furthermore, the Ca errors were both plus and minus. The Mg values were better, and there were plus as well as minus errors. In only two cases were poor Mg results obtained from Ca filtrates of pH 3; the Ca in these two instances had been precipitated with the unusually large excess of oxalate of 275 per cent. Therefore, a large excess of oxalate should be avoided, for, although correct Ca results may be obtained, the Mg values may be as much as 40 per cent too low.

*Determination of Ca, Mg, and  $\text{PO}_4$ —*Solutions analogous to those

described in the preceding two sections were then analyzed for calcium; phosphate was determined in the Ca filtrates in half of the analyses, and Mg was determined in the Ca filtrates from the other analyses. When Ca was precipitated at pH 6.3, the Ca values were high and the  $\text{PO}_4$  and Mg values were low. When the pH was adjusted at 3, excellent results were obtained for Mg, con-

TABLE I

*Analysis of Ca, Mg, and  $\text{PO}_4$  in a Mixture*

pH of Ca precipitation = 3.0.

Constituent		Weight of precipitate			Precision	
		Calculated	Found	Deviation	Reproducibility	Accuracy
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
Ca	0 3653	0 3658	+0.0006			
		0 3646	−0.0006			
		0.3664	+0.0012			
		0 3639	−0.0013			
Average.....		0.3652	±0 0009	0 2	0.0	
PO <sub>4</sub>	0.3837	0.3825	−0.0004			
		0.3826	−0 0003			
		0 3825	−0.0004			
		0.3840	+0.0011			
Average.....		0 3829	±0.0006	0.2	0.2	
Mg	0.0307	0.0321	+0.0003			
		0.0323	+0.0005			
		0.0316	−0.0002			
		0.0310	−0.0008			
Average....		0.0318	±0.0005	1.6	3.6	

sidering the small amount of Mg involved; excellent results were also obtained for Ca and  $\text{PO}_4$ .

A series of analyses was then performed in quadruplicate, the pH being regulated at 3.0 in the calcium precipitation. The results are summarized in Table I. Single precipitation was employed in the calcium determination and double precipitation in the Mg and  $\text{PO}_4$  determinations. The reproducibility of the Ca determination was 0.2 per cent and the accuracy was better

than 0.1 per cent; the reproducibility of the  $\text{PO}_4$  determination was 0.2 per cent, and the accuracy was also 0.2 per cent. In the case of the Mg determination the average deviation was smaller ( $\pm 0.5$  mg.) than that of the  $\text{PO}_4$  determination ( $\pm 0.6$  mg.); however, since the amount of magnesium taken for analysis was so small, the reproducibility in per cent and the per cent error were comparatively large. The reproducibility and accuracy of the magnesium determination were satisfactory for our purpose since they affected the final results in bone analyses to about the same extent as the deviations and errors in the calcium and phosphate determinations.

*Contaminants of Ca Precipitate*—The nature of the contaminants present in Ca precipitates obtained at pH 6.3 and in more alkaline solution, and absent when precipitation was carried out at pH 3.0, was investigated further.

Without the addition of oxalate, another aliquot of the mixture of Ca, Mg, and  $\text{PO}_4$  analyzed in Table I was brought to the purple color of brom-cresol purple (pH about 6.5); a gelatinous precipitate was obtained. This precipitate, obtained in the absence of oxalate, was found by colorimetric test to contain phosphate, as was to be expected.

In a series of seven analyses of the solution containing Ca, Mg, and  $\text{PO}_4$ , the Ca was precipitated as oxalate at pH 3 and filtered; colorimetric tests, with the method of Fiske and Subbarow (1925), showed that these precipitates contained no phosphate.<sup>5</sup> The filtrates were then made purple to brom-cresol purple. Additional precipitation occurred; the amount of precipitate obtained varied up to 46 mg., depending upon the amount of  $\text{NH}_4\text{OH}$  added. Colorimetric tests showed that both  $\text{PO}_4$  and Mg were present in the additional precipitate. Furthermore, when the filtrates were analyzed for Mg, all seven values were low; the errors ranged from  $-8.7$  to  $-91$  per cent.

These results, together with the data summarized in preceding sections, show that the cause of the high Ca values and of the corresponding low  $\text{PO}_4$  and Mg values, obtained when the Ca is precipitated from solutions that are insufficiently acid, is the

<sup>5</sup> The oxalate present in such a test does not prevent the development of the blue color when phosphate is present; it only delays it. (Personal communication from Dr. Fiske.)

contamination of the calcium oxalate precipitate with Mg and  $\text{PO}_4$ .

*Effect of Other Substances*—The effect of various substances on these determinations was next investigated. This aspect of the analytical problem was not exhaustively studied; the effect of a few added substances was determined in only a preliminary fashion to get some idea of the difficulties which would be encountered on analyzing solutions made from bones.

Fluoride is present in bones in small but significant amounts, according to a number of analysts. Although there is some question as to whether fluoride would be found in solutions made by extracting powdered bone with dilute HCl, a series of analyses was made on the mixture of Ca, Mg, and  $\text{PO}_4$  to which  $\text{NH}_4\text{F}$  had been added. Several preliminary precipitation tests were first made as follows: 2 mg. of F (as  $\text{NH}_4\text{F}$ ) were added to 10 mg. of Mg in one beaker and to 50 mg. of Ca in another beaker; the volume was about 100 cc. in both cases. When the pH was brought to 8.5, a slight precipitate was obtained in both solutions; but when in other aliquots the pH was adjusted to 3, no precipitate was obtained in either solution.

This showed that  $\text{MgF}_2$  and  $\text{CaF}_2$  did not precipitate at the pH which we used to obtain  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ . Mixtures of Ca, Mg, and  $\text{PO}_4$  were then analyzed for all three constituents after adding  $\text{NH}_4\text{F}$ ; it was found that  $\text{F}^-$ , up to a concentration of 2 mg. in the portion analyzed, did not affect markedly any of the determinations.

The addition of citrate is often recommended in the determination of Mg. When citrate was added, it was found that low Mg results were obtained under conditions which gave complete recovery in the absence of citrate. Good results were obtained for Mg in the presence of citrate provided the excess of  $\text{NH}_4\text{OH}$  was much larger than that used in the absence of citrate; about 2 or 3 times the usual excess was required. The calcium determination was not affected by the presence of citrate.

It was also found that small amounts of  $\text{Fe}^{+++}$  (up to a concentration of 1.5 mg. in the portion analyzed) did not interfere with the calcium analysis when precipitation was performed at pH 3. The iron was, however, precipitated with the phosphate and caused a distinct error; the iron imparted a yellow color to the

otherwise white precipitate. Citrate has been advocated by McCrudden (1909-10) for holding small amounts of iron in solution in such cases; we did not investigate this point further.

*Effect of Proteins*—In a few instances, solutions of bone salts were made by extracting bone powder with HCl. The physical properties of the solutions as well as of the precipitates showed that the erratic results which were being obtained were due to the presence of dissolved organic matter in the bone solution.

Some preliminary experiments on the effect of added proteins were thereupon performed. It was found that as little as 0.1 cc. of blood serum in the portion analyzed caused an appreciable error in the Ca determination; serum also affected the  $\text{PO}_4$  and Mg determinations. Dissolving and reprecipitating the Ca precipitates reduced the amount of the error, but even after reprecipitating twice, the Ca values were much too high.

Gelatine, in the quantities used (5 and 10 mg. in the portion analyzed), did not affect the determinations as serum did; the Ca errors were all small and were all negative.

Since trichloroacetic acid is widely used as a protein precipitant, the effect of adding it to the synthetic bone salt solution was investigated. When 45 and 70 cc. of a 10 per cent solution of this acid were contained in the portion analyzed, the analyses were not affected.

#### *Glass Filter Crucibles*

*Loss in Weight*—With the exception of a few instances in which Gooch crucibles were used, glass filter crucibles were employed in all the analyses reported here. The type we found convenient was the Jena crucible with the capacity of 30 cc. and a diameter of about 3 cm. These crucibles, with filter plates of sintered glass of various porosities, were found entirely satisfactory. The No. 3 crucibles were used for filtering the magnesium ammonium phosphate precipitates and the No. 4 crucibles, which were less porous, were used for the finer-grained calcium oxalate precipitates.

The only important source of error in the use of these crucibles found in the course of this investigation was the large loss in weight regularly sustained by some crucibles. The glass is appreciably soluble in the reagents employed in these analyses; the loss in weight ranged from 0.1 mg. to as high as 3 mg. in the course

of a single determination. It was found that some crucibles lost only a few tenths of a mg. regularly, while others regularly lost about 1 mg. in the course of each analysis. The change in weight is due to the solvent effects of the solution filtered, of the

TABLE II  
*Analysis of Final Standard Solutions*

pH of Ca precipitation = 3.0.

Constituent	Calculated	Pure solution		Mixture of Ca, Mg, PO <sub>4</sub>	
		Weight of ppt.	Deviation	Weight of ppt.	Deviation
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Ca	0.3924	0.3915	-0.0003	0.3927	-0.0010
		0.3907	-0.0011	0.3933	-0.0004
		0.3931	+0.0013	0.3946	+0.0009
		0.3918	±0.0009	0.3939	+0.0002
				0.3936	-0.0001
				0.3941	+0.0004
				0.3937	±0.0005
Reproducibility . . . . .		0.2 per cent		0.1 per cent	
Accuracy . . . . .		0.2 " "		0.3 " "	
PO <sub>4</sub>	0.3723	0.3721	+0.0009	0.3711	0.0000
		0.3704	-0.0008	0.3716	+0.0005
		0.3710	-0.0002	0.3705	-0.0006
		0.3712	±0.0006	0.3711	±0.0004
Reproducibility . . . . .		0.2 per cent		0.1 per cent	
Accuracy . . . . .		0.3 " "		0.3 " "	
Mg	0.0299	0.0296	0.0000	0.0304	+0.0002
		0.0296	0.0000	0.0300	-0.0002
		0.0295	-0.0001	0.0302	±0.0002
		0.0296	±0.0000		
Reproducibility . . . . .		0.0 per cent		0.7 per cent	
Accuracy . . . . .		1.0 " "		1.0 " "	

wash solutions, and of the acid and water used to clean the crucible on the completion of each analysis.

When the change in weight of the crucible was small, a crucible correction was made. One-half the loss in weight of the crucible was added to the weight of the precipitate to correct for this small

loss. Such a correction was quite satisfactory when the loss in weight amounted to only a small fraction of a mg. in each analysis; crucibles which regularly sustained larger losses in weight were rejected.

### *Final Standard Solutions*

As a final check on the methods, fresh standard solutions of Ca, of Mg, and of  $\text{PO}_4$  were prepared from an independent set of reagents. These new standard solutions were analyzed separately. They were then mixed and analyzed for Ca, Mg, and  $\text{PO}_4$ .

Table II gives these final results; the weights are those obtained by single precipitation in the case of Ca, and by double precipitation in the case of  $\text{PO}_4$  and Mg. The separate solutions were analyzed in triplicate; the reproducibility of the results in each case was excellent, and the mean value in each case agreed well with the calculated value. Aliquots of the three solutions were then mixed, and the mixtures were analyzed. Again the reproducibility and accuracy of the results were entirely satisfactory.

### RECOMMENDED ANALYTICAL PROCEDURES

As stated previously, the general procedures were modified in numerous ways. The conditions which were varied have been described in preceding sections. Many of them were found to be without important effect when varied between the limits stated. However, some of them resulted in errors of 1 per cent or more even when large amounts of each constituent were being determined.

The reproducibility and accuracy of the results given in Tables I and II were entirely satisfactory. These later analyses were performed according to the procedures which had been found, in the earlier analyses, to give the best results. The recommended procedures may be summarized as follows:

#### *Determination of Calcium*

Make the solution red to thymol blue with HCl, and bring the volume to 100 cc. Add 0.5 M oxalic acid sufficient to give from 40 to 140 per cent excess of oxalate (*e.g.*, about 7 to 12 cc. for 100 mg. of Ca). Heat to boiling and add 1 M  $\text{NH}_4\text{OH}$ ,

slowly with stirring, until the solution is yellow. Digest for about 3 hours on the steam bath. Cool and filter on a prepared glass crucible. (The crucible is prepared by washing with dilute  $\text{HNO}_3$  and with  $\text{H}_2\text{O}$ , drying at  $110^\circ$  for 1 hour, cooling, and weighing.)

Use small portions of 0.1 M  $\text{NH}_4\text{OH}$  to transfer the precipitate to the crucible. Suck dry after each addition of wash solution. Dry<sup>4</sup> for 1 hour at  $105^\circ$ . Cool in a desiccator 1 hour and weigh.

#### *Determination of Phosphate*

Evaporate the Ca filtrate and washings to 100 cc. Add 6 M  $\text{HCl}$  until a clear, red solution is obtained. Add enough 0.5 M  $\text{MgCl}_2$  to give about 2 to 4 times the equivalent of the phosphate present (*e.g.*, about 10 cc. for 50 mg. of P). Add 9 M  $\text{NH}_4\text{OH}$  slowly, with constant stirring, until the indicator turns blue. Then add slowly an excess of  $\text{NH}_4\text{OH}$ , increasing its concentration by about 0.4 mol per liter (*i.e.*, about 5 cc. of 9 M  $\text{NH}_4\text{OH}$  additional). Make the volume to 125 cc. and let stand overnight.

Decant through an unweighed crucible and discard the filtrate if clear. Dissolve the precipitate with 1 M  $\text{HCl}$ , using a total of about 40 cc. Return the solution to the beaker, using about 60 cc. of water as wash fluid. Add enough 0.5 M  $\text{MgCl}_2$  to make the total Mg about twice the phosphate equivalent (*e.g.*, about 3 cc. additional  $\text{MgCl}_2$  for 50 mg. of P). Add methyl red. Then add 9 M  $\text{NH}_4\text{OH}$  slowly until the indicator turns yellow; continue the addition, drop by drop, with vigorous stirring, until the excess of  $\text{NH}_4\text{OH}$  is the same as in the first precipitation. Make the volume to 125 cc. and let stand overnight.

Filter through a prepared crucible. (The crucible is prepared by washing, successively, with dilute  $\text{HCl}$ , water, ethyl alcohol, and ether. It is then dried at  $37^\circ$  for 1 hour, cooled, and weighed.) Use the clear filtrate to transfer the precipitate to the crucible. Wash, successively, with three 5 cc. portions of cold<sup>6</sup> 0.04 M  $\text{NH}_4\text{OH}$ , three 5 cc. portions of alcohol,<sup>7</sup> and once with 5 cc. of ether,<sup>7</sup> using suction. Dry at  $37^\circ$  for 1 hour. Cool in a desiccator 1 hour and weigh.

<sup>6</sup> The  $\text{NH}_4\text{OH}$  is kept cold in the refrigerator.

<sup>7</sup> Redistilled over alkali.

*Determination of Magnesium*

Evaporate the Ca filtrate and washings to 75 cc. Add HCl until a clear, red solution is obtained. Add 3 M  $\text{H}_3\text{PO}_4$  in an amount 15 to 30 times the Mg equivalent (*e.g.*, about 2 cc. for 5 mg. of Mg). Add 9 M  $\text{NH}_4\text{OH}$ , slowly with stirring, until the indicator turns blue; then continue the addition slowly, until the excess of  $\text{NH}_4\text{OH}$  is the same as in the phosphate determination. Make the volume to 100 cc. and let stand overnight.

Decant through an unweighed crucible and discard the filtrate if clear. Dissolve the precipitate, using 30 cc. of HCl. Return the solution to the beaker, using 50 cc. of water as wash fluid. Add about a 10-fold excess of 3 M  $\text{H}_3\text{PO}_4$  (*e.g.*, about 1 cc. for 5 mg. of Mg). Add methyl red. Add 9 M  $\text{NH}_4\text{OH}$  slowly until the indicator turns yellow. The procedure from this point is the same as in the determination of phosphate except that the final volume is 100 cc.

## DISCUSSION

The literature contains numerous studies of the determination of Ca, or of Mg, or of  $\text{PO}_4$ , in mixtures. Satisfactory results have been obtained by many analysts for the single constituent under consideration, but, as has been pointed out by several investigators, this is accomplished by means of compensating errors. The determination of all three constituents in the same mixture, a procedure which reveals the existence of these compensating errors, has received comparatively little attention.

Hall (1928) recently reviewed the separation of Ca from Mg as oxalate and stated, "It is clear that chemists differ widely with respect to how much ammonium oxalate should be used to accomplish the best results." He confirmed the statement of Fresenius that more oxalate is required to precipitate Ca completely when Mg is present than is required in a pure Ca solution; however, too large an excess of oxalate in the Ca analysis prevents complete precipitation of magnesium ammonium phosphate in the subsequent Mg analysis.

Large quantities of ammonium salts are frequently added in the Ca determination to hold the Mg in solution. According to Hillebrand and Lundell (1929), "The correct amount of ammo-

nium chloride to have present is a problem, a large excess reduces the precipitation of magnesium and barium but at the same time retards the precipitation of calcium. . . ."

The presence of all this added salt increases the ionic strength of the solution and thereby increases its solvent effect. When, however, precipitation is carried out in a sufficiently acid solution, the Mg does not interfere with the Ca analysis; addition of a large excess of oxalate or of ammonium chloride is thereby rendered unnecessary. Our results confirm and extend the work of Shohl (1922) and of Chapman (1928) who found that Ca can be completely precipitated<sup>8</sup> at pH 4.0. At pH 3 no large excess of oxalate or ammonium chloride is needed, even in the presence of Mg. Furthermore, single precipitation suffices to give a pure precipitate of calcium oxalate.

The magnesium ammonium phosphate is also precipitated in as acid a solution as possible. In the determinations both of  $\text{PO}_4$  and of Mg, all the reagents are added in acid solution, then  $\text{NH}_4\text{OH}$  is added slowly with constant stirring. When precipitation begins, the addition of  $\text{NH}_4\text{OH}$  is slowed down to a drop at a time. The solution is not made strongly alkaline until the major portion of the precipitate has formed.

In contradistinction to practically all of the work on Mg and  $\text{PO}_4$ , in which ignition is employed in the belief that the precipitate is quantitatively transformed to  $\text{Mg}_2\text{P}_2\text{O}_7$ , we have weighed the precipitate directly. Jones (1916) recommended direct weighing as  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  and employed it in the analysis of biological material. This method was criticized by Kleinmann (1919) who raised objections to the technique used by Jones. We have found that direct weighing of the precipitate, without ignition, is capable of giving good results.

When the precipitate of  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$  or of  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  weighed about 0.3 gm., the reproducibility and accuracy of the results were 0.3 per cent or better. Since Mg is present in bone in much smaller amount than Ca or  $\text{PO}_4$ , a ratio of Mg to Ca was selected for the mixture which gave about 0.003 gm. of Mg in

<sup>8</sup> Shohl concluded that, "If the solution is more acid than pH 4.0 calcium oxalate is dissolved." However, Shohl did not determine the effect of acidities intermediate between pH 2.8 and 4.0. Chapman found that, "calcium precipitates completely at an acidity at least as low as pH 3.6."

the Ca filtrate; the Mg precipitate obtained weighed about 0.03 gm. The average deviation and the accuracy, expressed in gm., were about the same for such small amounts as for larger quantities; when expressed in per cent, the reproducibility and accuracy were of course larger for the small amounts of precipitate.

A large number of determinations were performed in which the precipitates were ignited and weighed, or were dissolved and titrated; the results, however, were not as satisfactory as those obtained by direct weighing of the precipitates. It had not been our intention to devise a generally applicable scheme of analysis, or to simplify current procedures. We were prepared to accept laborious and time-consuming procedures for the sake of better accuracy than that obtainable by the use of standard techniques. Fortunately, however, the best results were found to be given by simple procedures.

The methods herein described are applicable, without further modification, to the analysis of precipitates obtained from solutions containing only Ca, Mg, and  $\text{PO}_4$ . For the accurate analysis of bone solutions, however, the possible interference of small amounts of dissolved organic matter and of ions present in bone in smaller amount than Ca,  $\text{PO}_4$ , and Mg should be taken into consideration. Furthermore, in employing such analytical data for the determination of the constitution of the compound, or compounds, present in bone the possibility should also be considered that some of the calcium may have been combined with other inorganic or organic acids, and that some of the phosphate may have been present in bone in combination with organic material.<sup>9</sup>

#### SUMMARY

1. In solutions just yellow to thymol blue (pH 3) calcium is completely precipitated as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ .

2. Direct gravimetric estimation of calcium, by simple drying of the precipitated  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$  at  $105^\circ$  and weighing, gives results accurate to  $\pm 0.3$  per cent.

3. Complete separation of calcium from magnesium and from phosphate is effected by a single precipitation at pH 3.

<sup>9</sup> This latter possibility is being investigated by Fiske and Logan. (Personal communication from Dr. Fiske.) Dr. Pond, in a personal communication, stated that he has been studying several of the other possibilities.

4. Direct gravimetric estimation of phosphate, by washing the reprecipitated  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  with alcohol and with ether, drying at  $37^\circ$  and weighing, gives results accurate to  $\pm 0.3$  per cent.

5. Direct gravimetric estimation of magnesium as  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ , by a method analogous to the determination of phosphate, gives results having the same absolute accuracy, in mg., as the phosphate determination; for an amount of precipitate one-tenth that obtained in the phosphate determination this amounts to a relative accuracy of about 3 per cent.

6. With the procedures described here, the same reproducibility and accuracy are obtained on analysis of a mixture containing Ca, Mg, and  $\text{PO}_4$  as are obtained on analysis of separate solutions of Ca, of Mg, and of  $\text{PO}_4$ .

7. The bearing of this work on bone analysis is briefly discussed.

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# A MODIFICATION OF THE STODDARD AND DRURY TITRIMETRIC METHOD FOR THE DETERMIN- ATION OF THE FATTY ACIDS IN BLOOD SERUM\*

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None of the micro methods for the determination of blood lipids has been entirely satisfactory. Gravimetry, although employed extensively in macro analyses of tissue lipids, has proved unsuitable for micro determinations because non-lipid materials have contaminated the fats and because hydrolysis of neutral fats and oxidation of fatty acids during the process of drying have altered the composition of the lipids (Leathes and Raper, 1925). Nephelometric methods have been inaccurate because the degree of dispersion of the lipids varies with the temperature and with the proportions of different fatty acids and lipids (Bloor *et al.*, 1922; Bloor, 1928). The chylomicron technique (Gage and Fish, 1924-25) only includes lipids in limited physical states and has not yielded data analogous to the control determinations made by chemical methods (Knudson and Grigg, 1922-23; Schroeder and Holt, 1926; Rony and Mortimer, 1931; Bloor *et al.*, 1927; MacArthur, 1930-31).

In Bang's method (1918) after oxidation of the lipids with dichromate and sulfuric acid, the excess chromic acid has been determined quantitatively by iodometric titration. This oxidation reaction is not specific for lipids, and contaminating materials such as lipid solvents and organic impurities alter the final titration value. As the reaction is never complete, minute variations

\* The data in this paper are taken from the dissertation presented by E. B. Man in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1932.

in the duration of oxidation or in the temperature of the process alter the amount of oxidation. Different factors have been used by Bang (1918), Blix (1926), Bloor (1928), Staub (1931), and Katsura and Hatakeyama (1931).

Acidimetric titration of the fatty acids in blood was the basis of both the Stewart and White (1925) and Stoddard and Drury (1929) techniques. In each of these, the extracted fat was saponified with sodium hydroxide in the presence of alcohol. In the Stewart and White procedure the hydroxide for saponification was neutralized with an equivalent amount of acid (5 ml.) before titration of the fatty acids. The error introduced by the addition of these relatively large amounts of alkali and acid, makes the method theoretically unsound. The titration value may be elevated by the presence of non-lipid acids, such as the acids produced by the action of alkali on glucose extracted from blood by the alcohol-ether mixture (Long *et al.*, 1932; Himwich *et al.*, 1931; Himwich, 1932). In the Stoddard and Drury technique, these difficulties were eliminated by filtering and washing the fatty acids after they had been freed by saponification and acidification. In 1931, Stewart modified his method to include this filtration (Stewart *et al.*, 1931).

In the attempt to utilize the method of Stoddard and Drury it has been found necessary, in the course of a critical examination, to modify practically every step of the technique originally described.

#### *Discussion of Method*

*Extraction*—Wet extraction of blood fat is preferable to dry extraction since in the latter technique unsaturated acids including even oleic acid may be oxidized (Leathes and Raper, 1925) and the lipid may become relatively insoluble (Fowweather, 1926; Saxon, 1914).

Hot alcohol has been found to be the most effective single extracting agent in spite of many attempts to use such fat solvents as various concentrations of ethyl alcohol, ether, chloroform, ethylene dichloride, higher alcohols, and alkyl halides (Kumagawa and Suto, 1908; Channon and Collinson, 1929; Luden, 1918-19; Anderson, 1927; Eckstein and Wile, 1926; Marcus, 1928; Titus *et al.*, 1928; Clough and Johns, 1923; MacArdle, 1925; Bloor, 1915,

1928; Bloor *et al.*, 1922). Shimidzu (1910), with Kumagawa's (1908) alcohol extraction method, recovered higher percentage yields of fat when the blood was extracted with alcohol before saponification than when the blood was saponified prior to extraction of the lipid material.

Stoddard and Drury used the method of extraction which Bloor (1928) has developed. Into a mixture of 3 parts of alcohol and 1 part of ether the plasma is allowed to run slowly enough to insure the formation of a finely divided precipitate. By immersing the containing flask in boiling water the mixture is then heated to boiling for a few seconds. After cooling, the protein residue is filtered from the lipid extract. With this method, extraction proved incomplete even when meticulous care was used to obtain a fine precipitate and to immerse the flasks in a uniform manner. (Theorell (1930), Blix (1926), and Szent-Györgi (1924) have found it difficult to extract the lipids completely from blood.) In eight experiments 5.0 to 31.0 per cent greater quantities of fatty acid were recovered when plasma was refluxed 1 hour with alcohol-ether mixture than when aliquots of the same plasma were extracted by Bloor's method. After trying from  $\frac{1}{2}$  to 2 hour periods of refluxing with different aliquots of the same plasma, it was obvious that extraction for more than 1 hour did not increase materially the recovery of plasma lipid. Finally, to test the completeness of extraction 10 to 20 ml. of oxalated blood or serum were extracted in 2 ml. aliquots, the protein residues were combined, reextracted, and the fatty acid content of the second extract determined. Table I summarizes the lengths of time of second extractions and the percentage recoveries of fatty acids. The first extraction of serum yielded 97 to 98.2 per cent of the total serum lipid.

*Saponification*—In order to determine the completeness of saponification, a series of experiments was performed in which the length of time and the agents for saponification of Pfanstiehl "pure" lecithin were varied. From the data of Maclean (Maclean and Maclean, 1927), Levene (Levene, 1921; Levene and Simms, 1921), and Cousin (1903) it was assumed that a gm. molecule (805 gm.) of lecithin contained one equivalent weight of oleic and one of stearic acid. The preparation as purchased yielded only 63.6 per cent of the calculated phosphorus and 71.4 per cent of the

calculated milli-equivalents of fatty acid. The lecithin, after purification by the procedures suggested by Maclean (Maclean and Maclean, 1927), had 97 per cent of the theoretical phosphorus content. The phosphorus determinations were made by a modifi-

TABLE I  
*Comparison of Methods of Extraction*

Sample No.	Nature of material	Fatty acid from initial extraction	Serum in sample	Initial extraction		Subsequent treatment	Alkali used for saponification	Fatty acids from second extraction	
				Fractions extracted	Time of refluxing				
		m.-eq.	ml.	ml.	hrs.			m.-eq.	per cent of total
1*	Blood	11.4	24	2	2	Washed with cold alcohol and ether	NaOH	0.04	3.3
2	"	11.1	24	2	2	Boiled 1 min. with ether	"	0.04	3.3
3	"	9.4	24	2	2	" "	"	0.02	1.9
4	"	11.6	16	2	3	Suspended in thimble over boiling alcohol 1 hr., over boiling ether 1 hr.	"	0.03	2.9
5†	Serum	14.6	20	1	1	Refluxed with 3 parts alcohol, 1 part ether	"	0.04	2.6
6	"	13.4	20	2	1	Same as Sample 5	"	0.01	1.0
7	"	42.8	11	2	1	" " " 5	"	0.04	1.0
8	Blood	10.7	24	2	1	" " " 5	"	0.04	3.6
9	"	9.8	24	2	1	" " " 5	"	0.04	4.0
10	Serum	12.9	16	2	1	" " " 5	KOH	0.02	1.8‡
11	"	18.6	20	2	1	" " " 5	"	0.02	1.1‡
12	"	12.2	15	3	1	Refluxed in 3 ml. portions with 3 parts alcohol, 1 part ether for 1 hr.	"	0.03	2.7

\* Samples 1 to 4, 96 per cent ethyl alcohol was used in preliminary extraction.

† Samples 5 to 12, 3 parts of alcohol and 1 part of ether were used in preliminary extraction.

‡ A third extraction similar to the second extraction gave no appreciable yield of fatty acids.

cation of the Fiske and Subbarow (1925) method which will be described in a subsequent paper.

The methods of saponification and the percentage recoveries of the fatty acids of lecithin are summarized in Table II. Ebullition and evaporation were all conducted on a hot-plate at low heat.

TABLE II  
*Recoveries of Lecithin Fatty Acids after Use of Different Methods of Hydrolysis*

Sample No.	Hydrolysis						Weight of lecithin	0.02 N NaOH		
	0.1 ml. alkali	Length of re-action	HCl	Length of re-action	0.1 ml. alkali	Length of re-action		Theoretical titer	Experimental titer	Recovery of fatty acids
		hrs.		ml.		hrs.				
1	NaOH*	2					3.4	0.423	0.241	56.9
2	"	2					6.8	0.846	0.585	69.2
3	"	2					17.0	2.11	1.356	64.3
4	"	1	0.15†	1.0	NaOH‡	3	21.25	2.640	2.090§	79.2
5	"	1		0.1	Dilute NaOH	0.25	19.38	2.410	1.579	65.5
6	KOH	1	0.1	1.0	KOH	0.25	19.38	2.410	1.953	81.1
7	"	1	3.0	1.0			19.05	2.368	1.907	80.6
8	"	1	0.1	2.0			19.05	2.368	1.0	46.5
9	"	1	0.1	1.0	KOH	0.25	19.05	2.368	1.753	74.1
10	"	1					20.59	2.553	2.105	82.7
11	"	4					20.59	2.553	2.194	85.9
Lecithin added to alcohol, ether, and serum	"	1					16.46	2.048	1.685	82.3

\* Saturated solution of NaOH.

† Acid to phenolphthalein.

‡ Alkaline to phenolphthalein.

§ From here on each number is the average of triplicate determinations.

|| 50 gm. of KOH and 50 ml. of water.

From the results in Table II certain points in regard to saponification are evident: (1) potassium hydroxide is a better agent for saponification than sodium hydroxide, (2) hydrochloric acid does not increase the hydrolysis, (3) saponification for 1 hour with 0.1

ml. of potassium hydroxide solution makes possible the recovery of 82 per cent of the theoretical amount of fatty acids of lecithin. Saponification for 4 hours only increases by 3 per cent the yield of fatty acids.

Potassium hydroxide was superior to sodium hydroxide as a saponifying agent for lipids of blood serum as well as for lecithin. Different aliquots of serum were saponified in pairs with potassium hydroxide and with sodium hydroxide. The data are presented in the accompanying tabulation.

Subject	Milli-equivalents of fatty acid	
	KOH	NaOH
M. C. . . . .	11 3 14.1	10.4 12.3
V. L. M. . . . .	13.3 15 2	13 1 13 0
C. H. W. . . . .	9 6 12 1	8 7 11 2
R. B. . . . .	13 7 16 5	12.3 15 4

*Evaporation*—After the saponification the soaps are evaporated to dryness. All the alcohol must be volatilized because the subsequent isolation of the fatty acids depends on their insolubility in water. Traces of alcohol with the water might alter the insolubility of the fatty acids.

During the evaporation the fatty acids are subject to oxidation and the final stages, at least, should be carried out in the presence of an inert gas such as carbon dioxide or nitrogen. Prolonged heating in air even at a temperature of less than 95° increases the combining power of the fatty acids. Two aliquots of blood serum fatty acids, which were heated for half an hour after complete volatilization of the alcohol, required for neutralization 15.0 per cent more 0.02 N NaOH than two aliquots of the same serum fatty acids which were heated only long enough for complete volatilization of the alcohol. Eight experiments were performed in which two aliquots of serum fatty acids were evaporated to dryness in air and two under carbon dioxide. In every case the titer for the serum fatty acids evaporated under carbon dioxide was 4.5 to 14.9

per cent lower than the titer for the serum fatty acids evaporated in air. When nitrogen instead of carbon dioxide was bubbled through the solutions in five experiments, the titers for the duplicates evaporated in nitrogen were 0 to 7.5 per cent greater than for those evaporated in air.

With the present method, however, failure to use an inert gas does not produce a large error if the worker is careful to stop drying as soon as the last traces of alcohol have been removed. Also if the heat used in evaporation is less than 95°, drying occasionally may be somewhat prolonged with impunity. This was shown early in the use of this method when two sets of determinations were made in which one of the duplicates was dried longer than the other and yet the duplicates checked. In one of the experiments on the saponification of lecithin, the soaps were dried 15 minutes on the hot-plate after evaporation of the alcohol with no alteration in the percentage recovery. Furthermore, five aliquots of one serum, four aliquots of each of two other sera, checked within  $\pm 5.00$  per cent of the mean fatty acid content. Palmitic and oleic and linoleic acids added to the alcohol-ether mixture used for extraction of serum lipids were recovered with a maximum variation of  $\pm 5.00$  per cent of the theoretical value.

*Separation of Fatty Acids from Aqueous Mixture*—Little is known about the solubility of the serum fatty acids in water because the precise character of the fatty acids present in human blood is uncertain and the chemical properties of the highly unsaturated fatty acids have not been adequately investigated (Lewkowitsch, 1921). It was not possible to procure any linolenic or arachidonic acids for quantitative recovery experiments. Tangl (Tangl, 1930, 1931; Tangl and Berend, 1930, 1931) has recently published some articles concerning the presence of a water-soluble fatty acid (arachidonic) in blood. According to this author, the amount in the blood is about 5.0 per cent of the total fatty acids. This work of Tangl suggested that some fatty acids might be lost in the Stoddard and Drury technique when the fatty acids were filtered from the aqueous mixture. To investigate this point, the aqueous mixtures and sodium chloride washings of 23.2 ml. of serum were combined. The solution was made alkaline to phenolphthalein with potassium hydroxide, and was evaporated at low heat on a hot-plate to about 100 ml. After the residual liquid had cooled

it was acidified with hydrochloric acid, phenolphthalein being used as an indicator, and was extracted five times with ether. The combined ether extracts were washed with four small portions (10, 10, 5, 5 ml.) of water until the test for chlorides was negative. The ether was finally evaporated under carbon dioxide at a temperature lower than 50°. The residue was taken up in alcohol and titrated with 0.02 N sodium hydroxide. The titer for the washings less that for the blank was 0.335 ml. of 0.02 N sodium hydroxide. Therefore, the loss of soluble fatty acids is negligible when the

TABLE III  
*Quantitative Recoveries of Oleic Acid, Linoleic Acid, Palmitic Acid, and Lecithin*

Addition to 1.6 ml. of serum of				Titer of serum fatty acids	Titer of serum fatty acid plus added lipids	Titer of added lipids	Theoretical titer of added lipids	Recovery of added lipids
Oleic acid	Palmitic acid	Lecithin	Linoleic acid	ml.	ml.	ml.	ml.	per cent
mg.	mg.	mg.	mg.					
15.56	12.86	16.46		1.358	6.475	5.113	5.272	97.00
				1.367	6.448	5.086		96.60
				1.416	3.115	1.691	1.675	99.10
				1.433	3.107	1.673		100.00
1.632	1.320	4.24	3.01	1.204	2.250	1.034	0.980	94.70
				1.229	2.250	1.034		94.70
				1.463	2.060	0.568	0.538	105.5
				1.520	2.040	0.548		102.2
8.16	6.59	21.2		1.204	6.019	4.803	4.898	98.2
				1.229	6.039	4.823		98.5

usual quantity of serum (1.6 ml.) is used and when the procedures are carried out in air. From our experiments with theoretical amounts of fatty acids it is evident that stearic, palmitic, oleic, and linoleic acids are recovered quantitatively by this method (Table III). The quantity of unsaturated fatty acids which may be recovered is uncertain. Unless all operations are carried out in the presence of an inert gas the chemical constitution of the unsaturated fatty acids is markedly altered. Furthermore, even if they had been preserved by the use of an inert gas, many of the

unsaturated fatty acids would be lost in the process of washing with 5.0 per cent sodium chloride, because of partial solubility in water. There is also the possibility that the oxidation products of some of the unsaturated fatty acids may survive as substances which have base-combining value in the final titration.

*Extraction of Fatty Acids after Filtration*—Extraction from the crucibles by merely washing with three 3 ml. portions of boiling alcohol removed only a variable part of the fatty acids. Occasionally as much as 34 per cent would remain undissolved. This difficulty was overcome by extracting the Gooch crucibles in the Underwriter's extraction apparatus (Cary-Curr, 1912) for 1 to 2 hours. This method is simple, effective, and less time-consuming than that of repeatedly extracting the crucibles with small portions of alcohol. Periods of extraction of more than 1 hour did not yield sufficiently large (less than 1.0 per cent) additional amounts of fatty acid to warrant a longer period of extraction.

*Titration*—*Thymol blue*, the indicator selected by Stoddard and Drury, gives a somewhat sharper end-point for people who are able to detect delicate changes in color from yellow-green-blue to clear blue, than does phenolphthalein. Other indicators have been tried; all have proved less satisfactory than the two mentioned above.

*Calculations*—The formulæ are given at the end of the exact procedure. Since this titrimetric method determines the combining power of the fatty acids, but shows nothing about the molecular weights of the acids or the chemical combinations in which these acids occur, they may be expressed accurately only as milli-equivalents of fatty acid. This unit has not been employed previously for lipids. Comparison of milli-equivalents of fatty acids with the lipid determinations of other investigators may be made easily. For example, the amount of tripalmitin which contains one equivalent weight of palmitic acid is one-third the molecular weight of tripalmitin  $\left(\frac{806}{3} = 269 \text{ gm.}\right)$ . If milli-equivalents of serum fatty acid are multiplied by 269 the lipids are expressed in mg. of tripalmitin per 1000 ml. of serum.

It was found that 82.0 per cent of the fatty acids of lecithin was quantitatively recovered with the use of potassium hydroxide as saponifying agent, and only 65.0 per cent with sodium hydroxide

as saponifying agent. Similarly lower values were obtained when sodium hydroxide was used instead of potassium hydroxide for saponification of serum lipids. The data concerning completeness of saponification have been presented in Table II. If the fatty acid content determined in aliquots of the same serum saponified with these two different chemical agents is factored on the basis of 65.0 and 82.0 per cent yields of phospholipid fatty acids, the agreement between the determinations is remarkably close. For this reason, it has been assumed that the per cent of saponification of serum phospholipids and of lecithin is similar. No experiments concerning the amounts of saponification of serum phospholipid were attempted. These substances are extremely labile. Manipulations to separate serum phosphatides might change the chemical constitution and properties of the phospholipids. The results obtained would not be indicative of the completeness of saponification of the unaltered phospholipids in serum.

All data which will be presented subsequently are expressed in milli-equivalents of fatty acid calculated directly from the titer and also in milli-equivalents of fatty acids calculated from the titer plus 18.0 per cent of the theoretical phosphatide fatty acids. The latter figure is used to represent the total fatty acid content of serum. The correction is based on the hypothesis that in phospholipids one atomic weight of phosphorus is united with two combining weights of fatty acids as in lecithin and cephalin. This assumption is open to criticism because the chemical nature of the phospholipids in normal and pathological human blood is not known.

*Treatment of Blood*—The blood has been drawn with the precautions recommended by Peters and Van Slyke (1932). It was taken without stasis and was drawn and kept under mineral oil to prevent a shift in water balance between the cells and serum with a concomitant change in concentration of serum. The blood has been centrifuged and the serum separated from the cells immediately. Unless otherwise stated, the serum was precipitated in the alcohol-ether mixture within  $1\frac{1}{2}$  hours of the time when the blood was taken, to avoid the danger of abnormal lipolytic action in pathological bloods (Weil and Crandall, 1932; Joslin *et al.*, 1917; Crandall and Cherry, 1930-31).

It seemed possible that while the blood was being centrifuged

the lipid material might rise to the surface of the serum and might then be partially soluble in the oily layer. To answer this objection samples of the same lipemic blood were placed in one tube under oil and in another without oil. The bloods were centrifuged, the sera separated from the cells, and the fatty acid content determined in duplicate on each of the two samples of serum. The determinations were within the limits of experimental error (18.5 milli-equivalents of fatty acid in serum from blood under mineral oil, 19.2 milli-equivalents of fatty acid in serum from blood not under mineral oil).

TABLE IV

*Comparison of Serum and Plasma Values of Blood Samples Drawn under Oil and Saponified with NaOH*

Name	Case	Milli-equivalents of fatty acid			Amount of oxalate used per ml blood	Difference in serum and oxalate plasma	Difference in heparin and oxalate plasma
		Serum	Oxalate plasma	Heparin plasma			
					mg	per cent	per cent
E. G.	Normal	17 32	15 03		3 5	+13 3	
E. M	"	15 68	14 31		3 0	+8 8	
Ko.	"	14 20	12 83		3 5	+9 7	
B C.	"	15 98	14 83		2 0	+7 2	
Sh.	Hypertension		16 50	18 30	3 5		+9 6
Sho	"	20 00	18 81	18 92	3 5	+5 94	+0 11
Ca.	"	11 67		12 83			
Wa	Arthritis	10 19	19 08	9 88	2 0	-0 11	-0 20

Serum was selected instead of plasma because the addition of oxalate to blood to prevent clotting produces minor but definite and variable changes in concentration of plasma. Maclean states that oxalate may form an insoluble precipitate with some of the phospholipids (Maclean and Maclean, 1927). No quantitative data could be found concerning the rôle of cephalin in the coagulation of the blood (Howell, 1911-12, 1912, 1916-17). A table compiled by Knauer (Magistris, 1931) indicates that determinations on oxalated blood are definitely lower than those made on serum after coagulation, or on defibrinated blood. These observations are in keeping with our own, which are recorded in Table IV.

The differences between the values for serum and oxalated plasma may all be explained on the basis of a shrinkage in red blood cell volume due to the presence of oxalate (Osgood, 1926). These findings do not corroborate the statement in the literature that clotting increases the blood lipids (Jung and Wolff, 1923, quoted by Currie, 1924).

*Percentage Error of Method*—From Table I on extraction, it is evident that 1.07 to 2.75 per cent of the total serum fatty acids is not recovered. This is not included in the calculation of the percentage error because it is a constant loss.

*Calculated Percentage Error*—The maximum error acceptable between duplicates is 5.0 per cent because the end-point is only sensitive to 0.04 to 0.05 ml. of 0.02 N NaOH. This error includes the possible deviations in measurement of 2 ml. of serum, 50 ml. of extract, and 0.8 part of this extract for the fatty acid determination. The sum of these possible errors in measurement is 0.26 per cent.

The maximum calculated error of the method is, therefore,  $\pm 5.00$  per cent.

Experiments have been performed to recover oleic acid, palmitic acid, linoleic acid, and lecithin added to the alcohol-ether mixture which is used for the extraction of serum. Serum was added and the fatty acid content of the combined substances determined (Table III). The fatty acid content of the serum was determined on different aliquots. By the differences in fatty acid content of the aliquots with and without added lipid the quantitative recoveries of added lipid substances were ascertained. The fatty acid content of lecithin was calculated as an 82.0 per cent yield of the theoretical combining power of the fatty acids.

This theoretically calculated error of the method and these percentage recoveries of added lipids indicate a maximum percentage error of  $\pm 5$ . Four determinations in two instances, and five in another case, which were within  $\pm 5.0$  per cent of the mean also confirm this value. The comparative results obtained by two different workers on ten cases were all within  $\pm 6.0$  per cent, except two which differed by 13.6 and 18.0 per cent. It seems, therefore, that although in occasional instances the error is  $\pm 9.0$  per cent when two workers have checked each other, the usual maximal error of the method is  $\pm 5.0$  per cent.

*Method for Determination of Fatty Acids in Blood Serum*

*Reagents*—Paraffin oil, U.S.P., white.

Aldehyde-free 95 per cent alcohol (MacArdle, 1925). About 1500 to 2000 ml. of 95 per cent alcohol are refluxed with 70 to 100 gm. of potassium hydroxide for about 6 hours. The alcohol is distilled into a side arm flask, the outlet of which is protected by a calcium chloride tube to prevent the addition of water to the alcohol.

To test for aldehydes (Scott, 1922) 10 ml. of alcohol are diluted with an equal volume of water in a glass-stoppered bottle; 1 ml. of alkaline silver reagent is added; the bottle is closed and placed in a dark chamber for an hour. The liquid is now filtered, and the filtrate is acidified with  $\text{HNO}_3$  and a few drops of  $\text{HCl}$  are added. A precipitate of silver chloride indicates non-reduction of silver salt and consequently a negligible amount of aldehyde in the sample.

*Alkaline Silver Nitrate Reagent*—3 gm. of  $\text{AgNO}_3$  are dissolved in a little water in a 100 ml. volumetric flask; 3 gm. of pure  $\text{NaOH}$  are added, followed by 20 ml. of strong  $\text{NH}_4\text{OH}$  and the whole is diluted to 100 ml.

*Ethyl Ether, Peroxide-Free*—Ethyl ether, Mallinckrodt, U.S.P., is redistilled through an eight bulb pear column (King, 1929; Clover, 1922).

The redistilled ether may be tested for peroxide in the following manner. Ether is shaken with one-half its volume of 10 per cent potassium iodide slightly acidified with sulfuric acid. If more than a faint yellow color develops the ether must be rejected or repurified.

Alcohol-ether mixture, 3 parts of the alcohol to 1 part of the ether, of the quality described above.

Potassium hydroxide, C.P., 50 gm. dissolved in 50 ml. of water (Hertwig *et al.*, 1926).

Hydrochloric acid, C.P. (sp. gr., 1.1874 to 1.1944).

1.0 per cent phenolphthalein in 95 per cent alcohol.

Thymol-blue, 0.1 per cent aqueous solution of the sodium salt.

0.02 N sodium hydroxide prepared from a saturated solution of carbonate-free sodium hydroxide.

5 per cent  $\text{NaCl}$  solution neutral to methyl red.

Methyl red saturated solution in 50 per cent alcohol.

*Apparatus*—Underwriter's extraction apparatus consisting of coiled metal.

Condensers, 10 ml. Gooch crucibles, 250 ml. Erlenmeyer flasks (Cary-Curr, 1912, and Eimer and Amend catalogue for 1927, No. 22766).

Micro burette, graduated in 0.02 ml. with a capacity of 3 ml.

Filter paper, fat-free, either Schleicher and Schüll No. 589 "fat-free" or Whatman No. 43. Either of these contains enough fat so that twenty-five sheets, after 4 hours extraction with boiling alcohol and saponification of the alcohol extract, require about 1.5 ml. of 0.02 *N* NaOH to neutralize the fatty acids. Therefore, the blanks should be filtered through filter paper, and it is advantageous to extract the filter paper with alcohol prior to its use (Kumagawa, and Suto, 1908; Szent-Gyorgi, 1924).

*Extraction*—2 ml. of serum are added drop by drop to 25 ml. of alcohol-ether mixture (or if more serum is available 3 ml. are used in 70 ml. of alcohol-ether mixture) in a 250 ml. Erlenmeyer flask, while the mixture is agitated thoroughly to obtain a finely divided precipitate. Two or three glass beads are added to produce even boiling. The alcohol and ether are then allowed to reflux for 1 hour under a short coil Graham condenser. Corks for flasks should be wrapped in tin-foil. The extraction fluid is filtered through fat-free filter paper into a 50 ml. volumetric flask. After the protein residue and filter paper have been thoroughly washed the extraction fluid is allowed to cool to room temperature and is made up to volume. If the larger quantity of serum was used originally the volume should be made up to 100 ml.

*Saponification*—40 or 75 ml. of the alcohol-ether mixture are measured into a 125 ml. Erlenmeyer flask or 100 ml. beaker and 0.1 ml. of potassium hydroxide solution is added. The saponification is allowed to progress 1½ to 2 hours, while the solution boils gently, the containing vessel being heated on a hot-plate. It has been found advisable to start this heating with very mild heat as the ether shows a tendency toward rapid ebullition. Two or three thicknesses of wire gauze are often placed under the flask, one of which is removed after the rapid boiling has ceased. When the volume of solution has been reduced to 10 ml., a cover-glass is placed over the flask to prevent rapid evaporation and boiling is continued for ½ hour at least, to insure complete saponification

unless duration of heating has been at least  $1\frac{1}{2}$  hours. Then the flask is placed in an oven (temperature  $95-100^{\circ}$ ) and while a gentle stream of  $\text{CO}_2$  or nitrogen is run into it, evaporation is continued.

*Separation of Fatty Acids*—After all the alcohol has evaporated and the contents of the flask are thoroughly dry, 15 ml. of distilled water are added and the flask is reheated to form a homogeneous suspension of the soaps. 1 drop of thymol blue is added and sufficient concentrated hydrochloric acid to turn the indicator to a distinct pink color. The flask is then placed in the ice box to insure complete separation of the fatty acids. Generally the fatty acids are allowed to separate overnight.

*Filtration*—The fatty acids are then filtered without suction through a 10 ml. Gooch crucible containing a paper pulp mat. The preparation of the crucibles is a salient point in the technique. Stoddard and Drury ((1929) p. 745) give the following directions for the preparation.

“Use a Gooch crucible, smallest size (top 28 mm., bottom 18 mm. in diameter). Set the crucible in a rubber washer which fits over the top of a small funnel. Push the stem of the funnel through the hole in a rubber stopper and set the stopper in a suction flask (500 cc. capacity). Have ready a paper pulp emulsion, made by shaking up a piece of soft filter paper (such as Schleicher and Schüll black ribbon, No. 589) in 300 to 400 cc. of distilled water. Shake this emulsion vigorously and immediately pour some into the crucible while there is a strong suction on. Repeat until a layer about 1 mm. thick is formed. Tamp the layer down carefully all over with the end of a glass rod. Allow the larger masses of filter pulp fibres in the emulsion to settle out, and pour on successive amounts of the thin upper suspension of isolated shreds, keeping a strong suction on and tamping down occasionally, until the filter is dense enough to offer a definite resistance to the suction. Take the crucible out of the rubber washer and dry in an air oven at  $110^{\circ}$  for 15 minutes. Take out and allow to cool before using. . . . If the filtrate is not perfectly clear, put it through the crucible again. If the filtration does not start in a few minutes, change the crucible to the funnel in the filter flask and start the suction very gently, with a test-tube under the funnel.”

The flask and fatty acids are washed with 5 ml. portions of the 5 per cent sodium chloride solution until one washing yields with 0.05 ml. of 0.02 N sodium hydroxide a definite pink color in the presence of phenolphthalein.

The crucible is then suspended from an Underwriter's condenser over a 200 or 250 ml. wide mouth flask. The original flask is

washed with 20 ml. of hot 95 per cent alcohol (in three washings) and the alcohol is poured over the fatty acids in the crucible. The receiving flask is placed on a hot-plate at low heat and the alcohol allowed to reflux through the crucible for 1 to 2 hours.

*Titration*—The fatty acids dissolved in the hot alcohol; approximately 15 ml. are then titrated with 0.02 N sodium hydroxide with phenolphthalein or thymol blue (blue end-point) as an indicator. The titration must be completed while the flask is still hot or CO<sub>2</sub> will be absorbed. The admission of compressed air freed from CO<sub>2</sub> by passing through soda-lime and concentrated NaOH prevents some of the absorption of CO<sub>2</sub> from the air and makes the end-point more delicate.

*Blanks*—Blanks should be started with the process of extraction of the serum and carried through each step in the procedure.

*Calculations*—This formula applies to the use of 2 ml. of serum.

$$(1) \quad \frac{20}{1.6} (A - B) = \text{milli-equivalents of fatty acid}$$

A = titration in ml. of 0.02 N NaOH; B = titration of blank; 20 is the milli-equivalent content of the hydroxide solution; 1.6 represents the ml. of serum in the aliquot.

If phospholipid determinations are made simultaneously, the fatty acid values may be corrected for the fact that only 82.0 per cent of the phospholipid fatty acids seems to be determined.

The value obtained in Equation 1+18.0 per cent phospholipid fatty acids is considered to be equivalent to the total fatty acid content of the serum, as far as can be judged at the present time.

#### SUMMARY

After a discussion of the available methods for the measurement of the total fatty acids of the blood, the method of Stoddard and Drury is subjected to critical examination. On the basis of this study a titrimetric method for the measurement of serum fatty acids (exclusive of very unsaturated or water-soluble fatty acids) is described.

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# THE EFFECT OF THE INGESTION OF A LARGE AMOUNT OF FAT AND OF A BALANCED MEAL ON THE BLOOD LIPIDS OF NORMAL MAN\*

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The observed effects of a single fat meal on the level of the blood lipids in normal man are inconsistent. There is much uncertainty as to the normal range of blood lipids in the postabsorptive state, because data have been collected by the use of a variety of methods, each of which has a large experimental error. As most authors do not state the limits of error for their particular techniques, it is often impossible to compare their data with those of other studies on the same problem.

A detailed description of the technique used in this study for the measurement of the serum fatty acids has been presented in a previous paper (Man and Gildea (1932-33)). It is fundamentally a modification of Stoddard and Drury's volumetric method, and was found to have a limit of error of  $\pm 5$  per cent.

The lipid phosphorus was determined by a modification of the method of Fiske and Subbarow (1925).

## *Range of Serum Lipids of Normal Subjects under Postabsorptive Conditions*

Table I presents the range of serum lipid values of normal healthy individuals. These subjects were from 21 to 40 years of age, and were members of the staff or students of the university. The blood was drawn about 9.00 a.m. Each person had eaten nothing since the previous evening meal, had indulged in no

\* Some of the data in this paper are taken from the dissertation presented by E. B. Man in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Yale University, 1932.

exercise except walking around the building, and was not emotionally disturbed by the procedure.

A comparison of these normal values for serum fatty acids with the determinations of other investigators is inconclusive. Stoddard and Drury (1929) published no data on the serum lipids of

TABLE I  
*Serum Lipids of Normal Subjects in Postabsorptive State*

Date	Subject	Sex	Phospholipid fatty acids	Fatty acids from titer	Total fatty acids from titer + 18 per cent of phospholipid fatty acids	Non-phospholipid fatty acids
			<i>m -eq</i>	<i>m -eq</i>	<i>m -eq</i>	<i>m -eq</i>
Feb 16, 1932	C W	M	4 6	9 6	10 4	5 8
" 16, 1932	R B	"	6 5	13 6	14 8	8 3
" 23, 1932	V L M	F	6 5	14 8	16 0	9 4
" 23, 1932	E F G	M	5 6	12 4	13 4	7 8
" 24, 1932	H D	F	5 2	9 2	10 2	5 0
" 24, 1932	E M	"	5 8	12 4	13 4	7 6
Mar 15, 1932	"	"	6 1	13 8	14 9	8 8
" 2, 1932	H H	M	5 0	9 3	10 2	5 2
" 2, 1932	E C	"	5 0	11 4	12 3	7 2
" 14, 1932	E B G	F	5 8	12 0	13 0	7 2
Dec 15, 1931	M C	"	5 2	12 0	12 9	7 8
" 15, 1931	M H	M	5 0	12 4	13 3	8 3
Average			5 5	11 9	12 9	7 4
Minimum			4 6	9 3	10 2	5 0
Maximum			6 5	14 8	16 0	9 4
Normal values for total fatty acids according to Page <i>et al</i> (1930)						
Average					12 8	
Minimum					8 3	
Maximum					17 4	

fasting normal subjects. Stewart, Gaddie, and Dunlop (1931) seem to have made their analyses on whole blood and, therefore, their determinations are not comparable with those on serum. The data of Patterson (1927), Stewart and White (1925), and Nicholls and Perlzweig (1928) were obtained by use of the Stewart

and White method which, as has been pointed out previously by Man and Gildea (1932), is unreliable and gives higher values than any of the other methods. The determinations made by modifications of the Bang oxidative method show wide variations because the oxidation-reduction quotients used in the calculation of values vary considerably with each worker. Failure in some cases to state the quotient used in calculation limits the acceptability of data.

Bloor (1928) presented no figures on fatty acids in the plasma from human blood. Blix (1926), Staub (1931), and Stenberg (1929) worked with whole blood. Rony and Ching (1930) worked with dog blood. Page *et al.* (1930) gave minimum, maximum, and average values for fatty acids in the plasma of normal subjects. These are presented at the bottom of Table I as mg. and milli-equivalents of fatty acids. The latter figures have been calculated from the data on the assumption that palmitic and oleic acids are present in equal quantities in blood plasma. This assumption is based on the data which Bloor (1928) used for his calculations. Page stated that he followed Bloor's method.

Many values for normal serum lipids have been made by nephelometric procedures. Of these, the Bloor, Pelkan, and Allen (1922) modification seems to have been used most frequently. In Bloor's original article he compared the values of plasma fatty acids determined by Kumagawa and Suto and by his nephelometric procedures, but none of these comparisons were made on plasma. An average of the normal values for plasma fatty acids obtained by Bloor (1916) by Hiller, Linder, Lundsgaard, and Van Slyke (1924), and by McClure and Huntsinger (1928), with the nephelometric method is approximately 330 mg. per cent of fatty acid or 12.0 milli-equivalents of fatty acid, which agrees closely with the average obtained by Page and by us. If one could accept the values of many other workers with the nephelometric or Stewart and White methods, the range of normal would be between 7.0 milli-equivalents (190 mg. per cent) and 37.2 milli-equivalents (1000 mg. per cent). A similar variation was found in the normal group in this laboratory during the period that the method of Stewart and White was used.

*Effect on Serum Fatty Acids and Phospholipids of Ingestion of Large Amounts of Fat*

The majority of studies have been made with the nephelometric technique. Hiller, Linder, Lundsgaard, and Van Slyke (1924) in a review of the literature found only a few determinations on normal human subjects, and with the exception of those of Bang, these had been made with the ultramicroscopic technique. Bang (1918, pp. 104, 111) estimated the neutral fat of the blood in man and found only moderate rises, or none at all, after a meal of 100 gm. of butter, 250 cc. of cream, 100 gm. of meat, and 10 gm. of bread. Hiller, Linder, Lundsgaard, and Van Slyke reported only slight rises in plasma fatty acids after a meal of butter containing 1 gm. of fat per kilo of body weight. One out of six subjects showed a decrease. The average rise was 21.9 per cent. This is in marked contrast to the results obtained on dogs by all observers but the amount of fat fed to the normal subjects was much less per kilo of body weight than that fed to animals.

Since 1924 a number of studies have been made on the effect of a single fat meal on the blood fat in man. Many of these have been performed with the nephelometric technique with variable results. McClure and Huntsinger found the most marked rise in plasma fatty acids after the introduction of oleic acid by tube into the duodenum, but they also found a rise after a meal of carbohydrate and a little protein. A review of the recent observations of workers using the oxidative techniques of Bang (1918, p. 86) and Bloor (1928) or the method of Stewart and White (1925) indicates that little attention has been paid to the amount of fat fed in relation to the size of the subject. The effects described range from a decrease of 96 mg. per cent of fatty acid to a rise of 400 mg. per cent or 100 per cent above the fasting level (Blix, 1926; Nicholls and Perlzweig, 1928; Page *et al.*, 1930). These results tend to confirm the work of Hiller. Therefore, it appears that a fat meal usually produces some increase in the level of serum fatty acids, but that frequently there may be no change whatsoever, and in some cases even a decrease may occur. After a fat meal the blood fatty acids usually begin to rise at 2 hours, reach a maximum between 3 and 5 hours, and return to normal at 7 and 8 hours.

*Experiment*—It seemed probable that more uniform results

TABLE II

*Lipemias of Normal Subjects Following Ingestion of 3.54 Gm. of Fat per Kilo of Body Weight*

Date	Subject	Time	Phospho- lipid fatty acids	Fatty acids from titer*	Non-phos- pholipid fatty acids
1931			m.-eq.	m.-eq.	m.-eq.
Nov. 12	E. F. G.	Before breakfast	5.1	11.8	7.6
		2 hrs. after	6.1	16.2	11.2
		4 " "	6.8	16.8	11.2
		6 " "	6.7	16.4	10.9
		Difference	1.7	5.0	5.6
" 20	V. L. M.	Before breakfast	7.7	13.9	7.6
		2 hrs. after			
		4 " "	8.4	18.6	11.7
		6 " "	8.5	16.0	9.0
		Difference	0.8	4.7	4.1
" 25	E. B. M.†	Before breakfast	6.9	15.6	7.9
		2 hrs. after	8.2	18.3	11.6
		4 " "	7.8	19.8	13.4
		6 " "	8.8	22.5	15.3
		Difference	1.9	8.9	7.4
Dec. 1	R. R. S.†	Before breakfast	4.6	8.9	5.1
		2 hrs. after	5.0	11.4	7.3
		4 " "	4.8	13.8	9.9
		6 " "	5.0	13.1	9.0
		Difference	0.4	4.9	4.8
" 4	L. H. C.†	Before breakfast	7.0	15.6	9.9
		2 hrs. after	7.5	20.4	13.3
		4 " "	7.8	23.9	17.5
		6 " "	9.0	29.4	22.0
		Difference	2.0	13.8	12.1
" 7	E. B. G.	Before breakfast	6.2	13.2	8.1
		2 hrs. after	6.4	14.7	9.5
		4 " "	7.2	17.9	11.0
		6 " "	6.6	14.3	8.9
		Difference	1.0	4.7	2.9
" 11	G. B. C.†	Before breakfast	6.9	15.2	9.5
		2 hrs. after	6.6	18.3	12.8
		4 " "	7.3	24.2	18.2
		6 " "	7.6	19.9	13.7
		Difference	0.7	9.0	8.7

\* NaOH was used in saponification.

† These subjects disliked the meal and experienced much discomfort in eating the whole amount.

TABLE II—*Concluded*

Date	Subject	Time	Phospho- lipid fatty acids	Fatty acids from titer*	Non-phos- pholipid fatty acids
1931			<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>
Dec. 14	L. J. T.	Before breakfast	5.7	11.9	7.2
		2 hrs. after	6.1	22.4	17.4
		4 " "	7.2	27.8	21.9
		6 " "	7.3	27.6	21.6
		Difference	1.6	15.9	14.7
" 21	L. B. W.	Before breakfast	4.9	8.8	4.8
		2 hrs. after	4.9	11.8	7.8
		4 " "	5.6	12.6	8.0
		6 " "	5.4	13.3	8.7
		Difference	0.5	4.5	3.9
Average, <i>per cent.</i> .....			18		62
Maximum, " ".....			28		133
Minimum, " ".....			5		34

could be obtained if the amount of fat fed were proportional to the weight of each subject, and if some attempt were made to make the meals palatable. For these reasons we adopted a meal consisting of unsalted butter, 40 per cent cream, 2 to 3 slices of toast, 1 cup of coffee and no (or at the most 1 teaspoon of) sugar. Many of the subjects enjoyed eating this meal when it was made up as milk toast, but several did not like milk toast and ate the butter on the toast and drank the cream; the latter group did not find the meal palatable. Each subject received between 3.5 and 4 gm. of fat per kilo of body weight. These subjects were taken from among the group which had previously been used as normals for the studies of the postabsorptive state. They had had no food since the previous evening. Blood was taken before the fat meal, which was eaten between 8.30 and 9.30 a.m. Subsequent samples of blood were withdrawn 2, 4, and 6 hours later. The results are recorded in Table II. Every subject showed a marked rise in serum fatty acids, the average being 62 per cent, the maximum 133 per cent, and the minimum 34 per cent above the fasting level. There was a marked difference among the various individuals in their ability to ingest and to digest with comfort these large amounts of fat. Four subjects, noted in Table II, disliked the

meal and experienced much discomfort in eating the whole amount. Afterwards, they were slightly nauseated off and on during the day and in the following 12 hours suffered from a mild diarrhea. One subject not included in Table II was nauseated by the meal and 6 hours later vomited what appeared to be most of the butter and cream. The other subjects did not mind eating the meal and three of them actually liked it; one man even insisted that he would have enjoyed a larger amount of fat. No correlation seems to exist between the level of lipemia reached and the ability to digest the meal with comfort. In six of the subjects the apex of the rise was reached in 4 hours and in three it came after 6 hours.

The phospholipids were also determined as lipid phosphorus. The changes were not as great as those of the fatty acids, the average rise being 18 per cent, the maximum 28 per cent, and the minimum 5 per cent. Serum sugar rose slightly in only three subjects; in the others it tended to be slightly lower than it had been in the postabsorptive state.

Thus it appears that a fat meal of 3.5 to 4 gm. per kilo of body weight does produce a consistent rise of at least 34 per cent in the serum fatty acids of normal adults.

### *Effect of Standard Balanced Meal on Serum Fatty Acids*

A standard balanced meal was fed to normal people similar to those used for the large fat meal. This meal is described below.

#### *Breakfast Eaten by Normal Subjects*

	Protein	Fat	Carbo- hydrate	Calories
	gm.	gm.	gm.	
150 gm. grape fruit sections.....	1.2	0.3	10.5	49.5
100 " whole milk.....	3.3	4.0	5.0	69.0
50 " cream.....	1.1	20.0	1.5	190.0
15 " corn flakes.....	0.9	0.3	12.2	54.0
25 " bread.....	2.5	0.3	13.2	64.8
28 " butter.....	0.3	23.8		215.3
2 eggs.....	13.4	10.5		148.0
Total.....	22.5	59.2	42.4	790.6

None of the people experienced any difficulty in eating this breakfast. Only two samples of blood were taken, the first in the post-absorptive state and the second 3 hours after ingestion of the meal.

TABLE III

*Alimentary Lipemias of Normal Subjects. Blood Taken before and 3 Hours after a Balanced Breakfast Containing 59 Gm. of Fat*

Date	Time	Subject	Phospholipid fatty acids	Fatty acids from titer	Total fatty acids from titer + 18 per cent of phospholipid fatty acids	Non-phospholipid fatty acids
1938			m -eq	m -eq	m -eq	m -eq
Feb. 16	Before breakfast	C. W.	4.6	9.6	10.4	5.8
	3 hrs. after		5.2	12.1	13.0	7.9
	Difference		0.6	2.5	2.6	2.1
" 16	Before breakfast	R. B.	6.5	13.6	14.8	8.3
	3 hrs. after		6.1	16.5	17.6	11.5
	Difference		0.4	2.9	2.8	3.2
Mar. 23	Before breakfast	V. L. M.	6.5	14.8	16.0	9.4
	3 hrs. after		7.8	19.9	21.3	13.5
	Difference		1.3	5.1	5.4	4.1
" 23	Before breakfast	E. F. G.	5.6	12.4	13.4	7.8
	3 hrs. after		6.0	12.8	13.8	7.9
	Difference		0.4	0.4	0.4	0.0
Feb. 24	Before breakfast	H. D.	5.2	9.3	10.2	5.0
	3 hrs. after		5.5	13.4	14.4	9.0
	Difference		0.3	4.2	4.2	3.9
" 24	Before breakfast	E. M.	5.8	12.4	13.4	7.6
	3 hrs. after		7.2	20.4	21.7	14.5
	Difference		1.4	8.1	8.3	6.9
Mar. 2	Before breakfast	H. H.	5.0	9.3	10.2	5.2
	3 hrs. after		5.2	11.2	12.2	7.0
	Difference		0.1	1.9	1.9	1.8
" 2	Before breakfast	E. C.	5.1	11.4	12.3	7.2
	3 hrs. after		5.7	17.6	18.6	12.9
	Difference		0.7	6.3	6.4	5.7
" 14	Before breakfast	E. B. G.	5.8	12.0	13.0	7.2
	3 hrs. after		6.0	16.4	17.5	11.5
	Difference		0.2	4.4	4.5	4.2

This meal amounted to between 0.5 and 1.0 gm. of fat per kilo of body weight for each person. The weight of carbohydrate plus protein was slightly greater than the weight of the fat.

Even this meal produced a rise in the serum fatty acids in all

subjects but one, and in this person the meal was equivalent to only 0.5 gm. of fat per kilo. The maximum increase was +40 per cent and the average +21 per cent (Table III). Thus, in this group the ingestion of other foods with fat did not prevent the rise in serum fatty acids as has been claimed by some workers (McClure and Huntsinger, 1928; Bang, 1918, pp. 104, 111).

The phospholipids showed only a very slight increase, in most cases not beyond the limit of experimental error.

#### SUMMARY

The normal range of the serum fatty acids for man in the post-absorptive state is discussed and compared with values obtained with a modification of the Stoddard and Drury technique.

The ingestion of 3.5 to 4 gm. of fat per kilo of body weight by nine normal men and women is shown to produce a marked rise in serum fatty acids and a moderate increase in the serum phospholipids.

The ingestion of a balanced meal, containing at least 0.6 gm. of fat per kilo and with carbohydrate plus protein slightly exceeding the weight of the fat, is found to produce a rise in the serum fatty acids of normal men and women.

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## CEREVISTEROL, A STEROL ACCOMPANYING ERGOSTEROL IN YEAST\*

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(Received for publication, September 12, 1932)

It is known that several minor sterols occur with ergosterol in yeast. Those that are present in largest amount are zymosterol (MacLean, 1928) and  $\alpha$ -dihydroergosterol (Callow, 1931). Seven other sterols were reported by Wieland and Gough (1930), but at least one of these appears to be a mixture according to Callow. Cerevisterol was observed by one of us, E. M. H., (Bills and Honeywell, 1928) as a component of crude yeast sterol. Apparently no other workers have encountered it, with the possible exception of Ellis (1918) who extracted a crystalline substance, m.p. 250°, from the unsaponifiable matter of *Polyporus nigricans*.

Characteristic of cerevisterol is its high melting point, 265.3° (corrected), which distinguishes it sharply from other sterols. The impure cerevisterol which we originally described melted at 240°, and it was only after many recrystallizations that a product of constant melting point was obtained. Another property of cerevisterol is its extreme insolubility in hexane, and largely by taking advantage of this peculiarity we have been able to obtain a sufficient amount for careful purification and study.

### *Preparation and Purification*

Cerevisterol is contained in the mother liquors remaining from ergosterol manufacture, the procedure of which is briefly as follows (Bills, 1930, 1932): Dry yeast is extracted with acetone, and the lipid fraction thus obtained is saponified. From the soap a very crude mixture of all the yeast sterols is obtained. This is dis-

\* Presented before the meeting of the American Society of Biological Chemists at Philadelphia, April 29, 1932.

solved in a warm mixture of acetone and ether, which on cooling deposits crystals of crude ergosterol. This crude ergosterol contains zymosterol and probably  $\alpha$ -dihydroergosterol, but the cerevisterol, which does not readily crystallize in the presence of impurities, remains largely in the liquor. Hence it is to be expected that even the crudest commercial ergosterol does not contain appreciable amounts of cerevisterol. When the mother liquor is evaporated, additional crops of very crude ergosterol can be recovered. These contain increasing amounts of zymosterol, but scarcely any cerevisterol separates out until the final crop is obtained. Even so, a large part of the cerevisterol remains in the residual oily liquor. We add to this liquor an equal volume of hexane and set the mixture to stand for about a week at  $-20^{\circ}$ . An amorphous precipitate is obtained which consists mainly of cerevisterol and zymosterol, with a little ergosterol.

The crude cerevisterol (both the final ergosterol crop and the amorphous precipitate) was crystallized once or twice from acetone and many times from alcohol-acetone 1:3. It was necessary to keep track of the cerevisterol by melting point determinations. Sometimes the cerevisterol came out in the crystals, sometimes in the liquor, according to the sterols predominating. In the fractional crystallization, the fractions melting below  $200^{\circ}$  were separately fractionated, and the portions therefrom which melted above  $200^{\circ}$  were carried along with the main cerevisterol fractions. When a melting point of  $265.3^{\circ}$  was attained, the cerevisterol was considered pure, because no further change could be brought about by recrystallization. This product showed  $[\alpha]_{5461}^{25} = -57.4^{\circ}$  in chloroform. We obtained 10 gm. of pure cerevisterol from 4500 kilos of dry yeast (*Saccharomyces cerevisiae*).

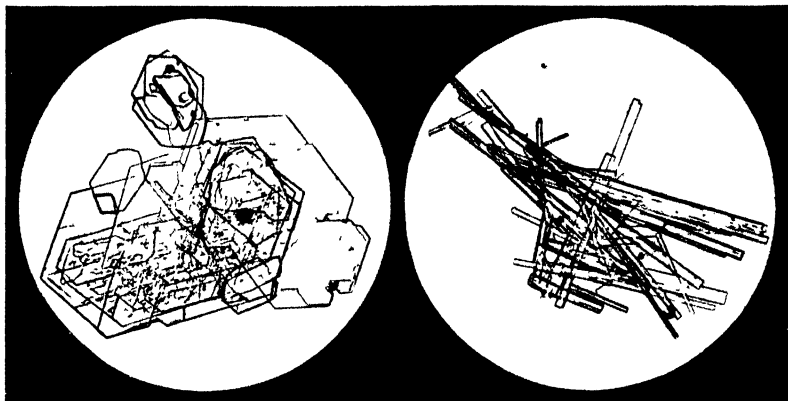
### *Properties*

Cerevisterol crystallizes in elongated prisms from alcohol or in broad hexagonal prisms from acetone or ethyl acetate. Measurement of a particularly good crystal from ethyl acetate showed the angles adjacent to the long sides to be  $124^{\circ}$ , and the angles between the short sides,  $112^{\circ}$ . Photomicrographs of the crystals are shown in Figs. 1 and 2.

The solubilities of cerevisterol, in comparison with ergosterol, are given in Table I. The figures are approximate, showing the

number of cc. of the common solvents at their boiling points required to dissolve 1 gm. of pure sterol. The presence of impurities greatly increases the solubility in most solvents and retards crystallization from supersaturated solutions.

The ultra-violet absorption spectrum of cerevisterol is entirely different from that of ergosterol, but it resembles that of isoergosterol in consisting of one band with a maximum at about 248  $m\mu$ .



FIGS. 1 AND 2 Cerevisterol crystals. About 60  $\times$ . Fig. 1 shows crystals from ethyl acetate; Fig. 2, from alcohol

*Solubilities of Cerevisterol and Ergosterol, Showing Approximately Volume of Boiling Solvent Required to Dissolve 1 Gm. of Sterol*

	Boiling point	Cerevisterol	Ergosterol
	$^{\circ}\text{C}$	cc. per gm.	cc. per gm.
Ether, $\text{C}_2\text{H}_5\text{O}$	35	1,000	70
Methyl acetate	54	500	35
Acetone	56	380	27
Methyl alcohol	65	300	280
Hexane	65-70	>100,000	24
Ethyl acetate	77	510	6.5
" alcohol (96 per cent)	78	250	50
Benzene	80	720	4.6
Isopropyl alcohol	82	100	10
Methylcyclohexane	101	2,200	<2
Alcohol-acetone (1.3)		250	

Its absorption is, however, only about one-eightieth as intense as that of isoergosterol. In Fig. 3 we show the absorption curve, as traced by a recording microphotometer from a photographic negative. Some cerevisterol was irradiated with ultra-violet rays

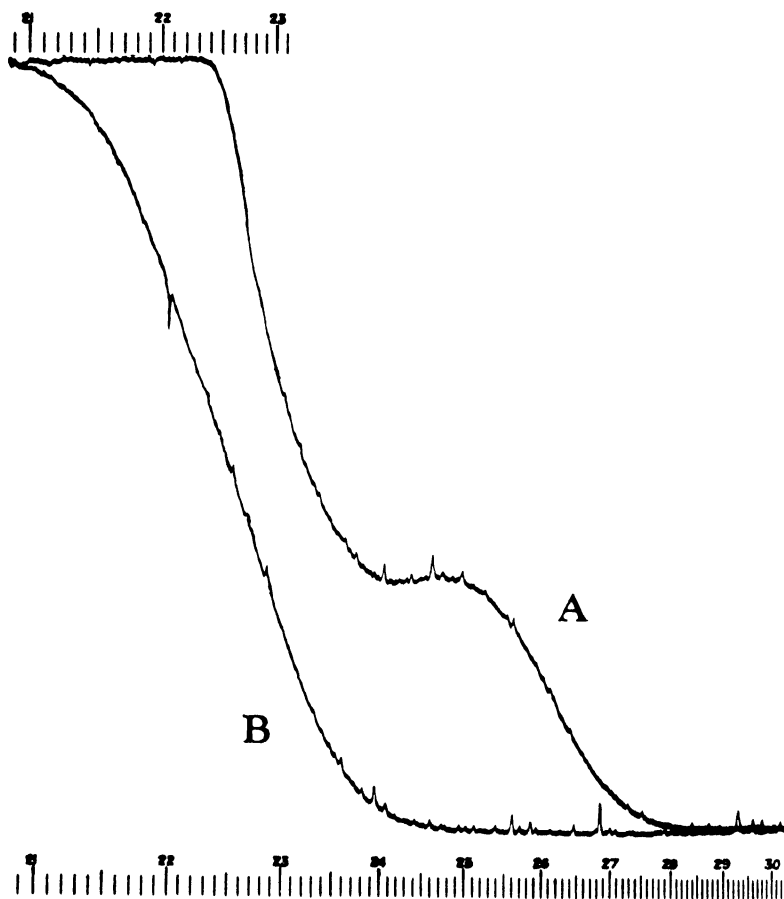


FIG 3 Recording microphotometer tracings of the ultra-violet absorption of (A) cerevisterol, (B) the base-line (photographic emulsion only) Concentration 0.5 mg. per cc. in alcohol; cell thickness 2 cm.

under conditions suitable for the activation of ergosterol. The product was administered to rickety rats in graded doses, the highest being 20 mg. per 100 gm. of food. This is 50,000 times the

healing dose of activated ergosterol, yet no healing resulted. This fact, together with the absorption spectrum, demonstrates the absence of even traces of ergosterol in the purified cerevisterol.

Cerevisterol is remarkably stable. Exposed to the air and light of the laboratory for several weeks, it showed no discoloration or change in melting point.

### *Chemical Composition*

Micro analyses of cerevisterol showed the following percentage composition, with only traces of ash.

Combustion No.	Analyst	C	H	O (by difference)
I	Research Service Laboratories	77.96	11.14	10.90
II	" " "	78.05	11.14	10.81
III	Dr. A. Schoeller, Berlin	77.95	10.70	11.35
IV	" " " "	78.06	10.65	11.29

These values correspond to the formula  $C_{28}H_{47}O_3$ . Comparison with the new formula for ergosterol,  $C_{28}H_{44}O$ , given by Windaus and Lüttringhaus (1932) indicates that cerevisterol has 2 more oxygen atoms than ergosterol and 3 more hydrogen atoms.<sup>1</sup>

The molecular weight of cerevisterol, as calculated from the formula, is 431.4. A determination by Rast's method, the melting point depression of *d*-camphor being employed, gave 415.3. This is about 4 per cent low, but it suffices to show that cerevisterol is not a condensed molecule such as an ether or a polymer.

Cerevisterol probably contains two double bonds. Like ergosterol and isoergosterol, it gives erratic iodine values, even with the Rosenmund-Kuhnhehn reagent. At 0° with this reagent one double bond is instantly saturated, and within 5 minutes about 50 per cent more halogen is taken up. At room temperature after 2 minutes an iodine value of 135 was obtained, as compared to the theoretical 118 for two double bonds and 177 for three double

<sup>1</sup> In the original abstract of this paper (Honeywell, E. M., and Bills, C. E., *J. Biol. Chem.*, **97**, p. xxxix (1932)), we gave the formula  $C_{28}H_{44}O_2$ . Such is the value calculated from Combustions I and II only. The more probable formula now presented,  $C_{28}H_{47}O_3$ , is calculated from Combustions I to IV inclusive.

bonds. Trebly unsaturated sterols, such as ergosterol and isoergosterol, absorb in 2 minutes at room temperature more than the theoretical amount of halogen required for three double bonds. It is a reasonable conclusion, therefore, that cerevisterol contains two double bonds.

Cerevisterol forms a diacetate, which indicates that 2 of the 3 oxygen atoms occur as hydroxyl groups. 1 gm. of cerevisterol was refluxed with 20 cc. of acetic anhydride for 5 minutes. The ester was precipitated by the addition of 80 cc. of 65 per cent alcohol and recrystallized from 80 per cent alcohol. The yield was 0.9 gm. of colorless needles. The ester melted clear at  $171^{\circ}$  and remained liquid until cooled to about  $124^{\circ}$ , when it became a translucent solid. The completeness of esterification was demonstrated by repeating the acetylation for 30 minutes instead of 5 minutes, the same product being obtained. Cerevisteryl diacetate showed  $[\alpha]_{5461}^{25} = -163^{\circ}$  in chloroform. While it is unusual for a sterol ester to exhibit greater optical activity than its parent sterol, a similar anomalous behavior is noted with isoergosteryl acetate and chloroacetate (McDonald and Bills, 1930). Saponification of the diacetate gave back cerevisterol of the original specific rotation and melting point.

The saponification value was determined as follows: 250 mg. of cerevisteryl diacetate were refluxed for 5 minutes with 25 cc. of 2 per cent alcoholic KOH. The product was titrated with 0.1 N HCl, with phenolphthalein as indicator. The completeness of saponification was demonstrated by repeating for 20 minutes instead of 5 minutes, the results being essentially identical.

#### *Saponification Value*

Theoretical for $C_{28}H_{46}O(C_2H_3O_2)_2$ .....	218
Found by 5 min. saponification.....	219
“ “ 20 “ “ .....	216

We have been unable to ascertain the nature of the 3rd oxygen atom in cerevisterol. Apparently it is not in an aldehyde or ketone group, since cerevisterol did not give any characteristic reaction product with 4,4-diphenylcarbazide, 2,4-dinitrophenylhydrazine, dimethyldihydroresorcinol, or thiobarbituric acid.

Cerevisterol gives the Salkowski and Burchard-Liebermann color reactions more strongly than cholesterol or ergosterol.

With the trichloroacetic acid reagent of Rosenheim (1929), its behavior is significant. The initial red color develops immediately. This, according to Rosenheim, is evidence of the presence of the  $\Delta^{1,2}$  (or  $\Delta^{1,13}$ ) ethenoid linkage which cerevisterol seemingly shares with ergosterol and with isoergosterol (McDonald and Bills, 1930). When very minute amounts of cerevisterol are used in this test, the initial red color soon fades and the solution becomes colorless. When larger amounts are used, the red color gives way to dirty green or even black, but this phase of the reaction is less intense than with ergosterol. The blue or greenish blue color given by ergosterol does not appear. It is therefore impossible to say whether the second double bond of cerevisterol occurs also in ergosterol.

#### *Other Minor Sterols of Yeast*

- Zymosterol was obtained from the sterol fractions which melted between 90–120°. Zymosterol is easily soluble in alcohol, but only sparingly soluble in ether, and we found that a mixture of alcohol-ether 2:1 was excellent for the recrystallization of this sterol.

From the fractions which melted between 120–155° a crop of fine crystals was obtained out of acetone. This product resembled the maize oil sitosterols in melting point and crystalline habit. In fact, we obtained a product seemingly identical with it from the unsaponifiable fraction of the sulfonated maize oil used as an antifoam in the culturing of the yeast. Since vegetable oils are almost universally used in the manufacture of aerated yeast and are sometimes also added to the finished press-cake, one should bear in mind the possibility of confusing foreign vegetable oil sterols with those of yeast itself.

From the fractions which melted between 155–180° ergosterol was obtained by recrystallization from alcohol-benzene 2:1.

The fractions which melted from 180–230° were separated by means of alcohol-acetone 2:1 into a less soluble fraction (crude cerevisterol) and a more soluble fraction which melted, impure, around 195°. The latter, probably crude  $\alpha$ -dihydroergosterol, was not further investigated.

The sterols of yeast which have been identified beyond reasonable doubt are ergosterol, zymosterol,  $\alpha$ -dihydroergosterol, and

cerevisterol. Low melting phytosterols from vegetable oil undoubtedly find their way into most commercial yeast, but whether the several sterols described by Wieland and Gough are related to these is not certain. Our method of separation was so different from that of the German workers that our failure to obtain evidence of numerous other sterols does not disprove their existence.

#### SUMMARY

Cerevisterol, a stable, hexane-insoluble sterol obtained from the mother liquors after the extraction of ergosterol from yeast, has the probable formula  $C_{28}H_{47}O_3$ . Highly purified, it melts at  $265.3^\circ$ , has  $[\alpha]_{5461}^{25} = -57.4^\circ$  in  $CHCl_3$ , and shows a weak absorption band,  $\lambda$  248  $m\mu$ . Irradiation does not activate it antiricketically. It has two double bonds and two hydroxyl groups. The nature of its 3rd oxygen atom is undetermined. The angles adjacent to the long sides of its crystals measure  $124^\circ$ ; those between the short sides,  $112^\circ$ . One of its double bonds is apparently the  $\Delta^{1,2}$  (or  $\Delta^{1,13}$ ) linkage of ergosterol and isoergosterol. Cerevisteryl diacetate melts at  $171^\circ$  and has  $[\alpha]_{5461}^{25} = -163^\circ$  in  $CHCl_3$ .

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# THE ANTIRICKETIC POTENCY OF EGGS FROM HENS RECEIVING MASSIVE DOSES OF ACTIVATED ERGOSTEROL\*

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(Received for publication, September 12, 1932)

It is known that the antiricketic activity of egg yolk is influenced by the content of vitamin D in the ration of laying hens, and the exposure of the birds to ultra-violet light (1-4). However, no data are available on the efficiency of transfer of the vitamin in massive doses.

For this study eggs were first gathered from fifteen Leghorn pullets receiving a normal ration compounded as follows:

Ground yellow maize.....	49
Wheat bran.....	10
Wheat middlings.....	10
Ground oats.....	10
Protein concentrate (consisting of maize protein 55, whole dried milk 10, meat scraps 35).....	12
Alfalfa meal.....	2
Iodized table salt.....	1
Calcium carbonate.....	4
Cod liver oil.....	2

The birds were kept indoors away from sunlight, and they received this ration exclusively from January 20 to March 17. Eggs were collected for assay during the last week.

On March 18 the 2 per cent of cod liver oil in the diet was replaced by 2 per cent of a maize oil solution of activated ergosterol having a cod liver oil coefficient of 10,000 by assay on rats (1000 international units per mg. of solution). After the birds

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had received this massive dosage of vitamin D for 6 weeks the eggs were again collected for 1 week, and prepared for assay.

The eggs were placed in boiling water for 10 minutes, and the yolks were removed, dried in a vacuum oven at low temperature, and extracted with ether. The ether was removed under a vacuum and the yolk oil was administered to ricketic rats according to the technique developed in this laboratory (5). The birds were weighed weekly during the period of administration of activated ergosterol, and a record was kept of the number of eggs produced during the two periods.

The protocols revealed that the egg oil from the birds which had received 2 per cent of cod liver oil for 7 weeks had a cod liver oil coefficient of 0.7, while that from the same birds after receiving 2 per cent of 10,000  $\times$  activated ergosterol solution for 6 weeks showed a coefficient of 130. The probable error of assay was  $\pm 8$  per cent. This represents an increase in potency of 185 times.

The fact that an increase of 10,000 times in dosage of vitamin D resulted in an increase of only 185 times in the antiricketic value of the egg indicates that the birds did not transfer the vitamin to the yolk efficiently. A similar inefficiency of transfer is shown by the milch cow, since only relatively small increases in the potency of the milk have been observed after large quantities of irradiated ergosterol and irradiated yeast were given (6-8).

During the period of administration of activated ergosterol the pullets lost about 20 per cent in weight and the general appearance was poor. They produced in the 7 weeks 160 eggs, a daily average of three and three-tenths eggs for the group. In the preceding 56 days when the birds received 2 per cent of cod liver oil, an average production of seven and three-tenths eggs per day was recorded. This decrease in weight and production was, no doubt, caused by the excessive quantities of vitamin D ingested.

On continuing the administration of 2 per cent of 10,000  $\times$  activated ergosterol solution for an additional 44 days (*i.e.*, May 6 to June 19), a further decrease in weight and egg production was noted. In fact, three of the birds died, and only forty-three eggs were laid.

Since it is known that irradiated ergosterol and irradiated yeast are less effective than the rat-equivalent amount of cod liver oil

in the prevention of leg weakness in chickens (9-12), it becomes important to investigate the effectiveness of the vitamin D transferred to the egg in the prevention of this condition. Day-old chicks were divided into three groups of fifteen each, and placed in suitable brooders in a darkened room. Tap water was given *ad libitum*. They received the following basal diet, in which was incorporated the oil containing vitamin D.

Yellow maize.....	41	Oats.....	10
Skim milk powder.....	15	Iodized table salt.....	1
Wheat middlings.....	10	Calcium carbonate.....	1
Wheat bran.....	10	Oil to be tested.....	2
Linseed oil meal.....	10		

Group 1 received the basal ration with 2 per cent of cod liver oil. Group 2 received the basal ration with 2 per cent of the high potency yolk oil suitably diluted with maize oil so as to be the

TABLE I  
*Showing Bone Ash of Birds Receiving Cod Liver Oil in Comparison with Egg Yolk Oil*

Group 1. Basal ration with 2 per cent cod liver oil		Group 2. Basal ration with 2 per cent maize oil containing egg yolk oil equivalent to 2 per cent cod liver oil		Group 3. Basal ration with 2 per cent maize oil containing egg yolk oil equivalent to 25 per cent cod liver oil	
Bird No.	Bone ash <i>per cent</i>	Bird No.	Bone ash <i>per cent</i>	Bird No.	Bone ash <i>per cent</i>
1	42.96	16	38.20	31	42.00
2	45.00	17	39.10	32	41.67
3	46.00	18	41.02	33	42.00
4	44.60	19	36.96	34	38.72
5	45.21	20	37.03	35	39.21
6	44.20	21	39.21	36	44.09
7	42.81	22	39.60	37	42.07
8	43.96	23	Died	38	39.97
9	44.02	24	"	39	Died
10	43.96	25	"	40	"
11	44.62	26	"	41	"
12	42.06	27	"	42	"
13	47.00	28	"	43	"
14	46.02	29	"	44	"
15	Died	30	"	45	"
Average...	44.46		38.73		41.22

equivalent of 2 per cent of cod liver oil by assay on rats. Group 3 received the same as Group 2, except that the yolk oil was diluted so as to be equivalent to 25 per cent of cod liver oil. Rations were made up weekly to eliminate possible loss of potency due to oxidation.

The birds were kept on the three diets for 7 weeks. The femurs were then removed, cleaned thoroughly, crushed, extracted with alcohol and ether, and ashed. The percentage of ash was calculated on the basis of the dried extracted bone. The findings are shown in Table I.

Table I reveals that yolk oil in doses equivalent to 2 per cent, or even to 25 per cent, of cod liver oil (by assay on rats) was much less effective than 2 per cent of cod liver oil for preventing leg weakness in chickens. It should be kept in mind that irradiated ergosterol was the original source of the vitamin D available to the pullets from which the yolk oil was obtained. This leads to a study to determine whether the source of vitamin D available to the laying bird determines the antiricketic value of the vitamin D that will be stored in the egg. Some experiments to investigate this point are being conducted.

#### SUMMARY

1. The egg yolk oil from pullets receiving 2 per cent of cod liver oil had a cod liver oil vitamin D coefficient of 0.7. The substitution of an irradiated ergosterol solution 10,000 times as potent as the cod liver oil resulted in an increase of only 185 times in the vitamin D potency of the yolk oil, as measured by tests on rats.

2. The egg yolk oil from the pullets receiving the massive dose of irradiated ergosterol was administered to baby chicks at levels equivalent to 2 per cent and to 25 per cent of cod liver oil, as measured by tests on rats. It was less effective at these levels than 2 per cent of cod liver oil.

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# CHANGES IN PHOSPHORIC ESTER CONTENT OF THE RED BLOOD CELLS AND THE LIVER IN EXPERI- MENTAL RICKETS

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That the inorganic phosphate content of the blood or plasma is greatly diminished in the large majority of cases of both human and experimental rickets has been known for some years. There is, however, little information available with regard to possible quantitative changes in other phosphorus compounds in the blood and the tissues, with which compounds the inorganic phosphate of the plasma is presumably in equilibrium. It was reported by Zucker and Gutman (1) that despite the fall in the inorganic phosphate content of the plasma or of the whole blood, the quantity of acid-soluble phosphorus (which includes the inorganic) is not diminished in rickets. (Therefore the *organic* acid-soluble phosphorus must be increased.) György (2) reported similar findings. Heymann (3) in a very interesting investigation finds that if children are given a phosphorus-poor diet for 4 days, their plasma inorganic phosphate diminishes, but that this diminution is accompanied by a rise of similar magnitude in the acid-soluble organic phosphorus of the plasma. In a very recent paper Stare and Elvehjem (4) report a small but definite decrease in ester phosphorus in the whole blood of rachitic calves, though it is not quite clear from their numerical data whether this may not have been due to a slight degree of anemia in their rachitic animals (5). In view of the phosphatase theory of the control of deposition of bone salts in ossifying cartilage, such findings are of considerable interest.

We have made several attempts to determine whether there is an increase in the acid-soluble organic phosphorus compounds in

the *plasma* in experimental rickets in rats, but so far, we have been unable to confirm or disprove it. The quantity of acid-soluble organic phosphorus in the plasma is in any case very small, and depends for its determination on the difference between two much larger quantities. Its accurate evaluation is not only rendered more difficult by the fairly rapid changes which are known to occur, as a result of enzymic activity, in the phosphorus compounds in recently shed blood, but is vitiated at once by a slight degree of hemolysis.

We have obtained, however, information which we believe to be definite concerning changes occurring in this disease in the quantity of acid-soluble phosphoric esters *in the red blood cells*, changes which may be associated with the pathogenesis of experimental rickets. There is also evidence, albeit rather less definite, in our experiments to indicate similar changes in the liver.

The present work began as a result of an observation made some time previously (6) that in severe human rickets there was usually, but not invariably, a rise in the phosphatase content of the blood plasma. It was expected that a similar state of affairs would be found in experimental rickets in rats. Such, however, was not the case. A number of experiments showed that the phosphatase value, not only of the plasma, but of other organs varied considerably from one group of rats to another, and that there was no significant difference between normal and rachitic animals in this regard. (The method used for the determination of phosphatase activity is that previously outlined (7), with slight modifications.)

A few experiments typifying these findings are given in Table I.

To summarize the experiments shown in Table I and many others not detailed here, the usual findings in experimental rickets are a slight fall in the kidney phosphatase, a slight rise in the bone phosphatase, a fall in the intestinal phosphatase (upper 8 inches of the small intestine), and irregular changes in the plasma phosphatase.

At the same time the livers of some of the animals from each group were examined with regard to their content of acid-soluble phosphoric esters and it was observed that those organs from rats on normal diets contained definitely more phosphoric ester than those from rats on rachitogenic diets. Other tissues were subsequently examined, and the phenomenon was found to be a fairly

general one. The effect is particularly noticeable in the red blood cells.

TABLE I  
*Changes in Phosphatase Content of Tissues in Experimental Rickets*

Diet*	Days on diet†	x-Ray and bone ash findings‡	Phosphatase content (arbitrary units) per gm. tissue or cc. plasma				
			Plasma*	Bone	Small intestine	Whole kidney	Liver
Normal, stock	25	Normal	0.69	36	43	38	1.0
Steenbock Ration 2965 (8)	25	Rickets +++	0.51	41	41	29	0.9
Normal, stock	25	Normal	0.60	40	85	57	1.3
McCullum Diet 3143 (9)	25	Rickets ++	0.56	30	70	48	0.9
Normal, stock	31	Normal	0.48	60	41	28	
Steenbock Ration 2965	31	Rickets +++	0.79	49	27	21	
High phosphorus	31	Osteoporosis	0.75	42	29	24	
Normal, stock	27	Normal	0.64			49	
Steenbock Ration 2965	27	Rickets ++	1.14			47	
Same + cod liver oil	27	Very slight rickets	1.04			46	
" + viosterol	27	Normal	0.99			46	
Normal, stock	29	"	0.99			43	
McCullum Diet 3143	29	Rickets +++	0.61			30	
Same + cod liver oil	29	Very slight rickets	0.69			42	
" + viosterol	29	Normal	0.51			43	

\* 5 per cent of brewers' yeast was added to both the Steenbock and the McCullum diets in all experiments recorded in this paper.

† Animals were usually 22 to 24 days old when put on the diet.

‡ +++ = severe; ++ = fairly severe.

### Methods

*Acid-Soluble Phosphoric Esters of Liver, Kidney, Spleen, Etc.*—The animal is quickly anesthetized with ether, and bled out from a jugular vein into a tube containing potassium oxalate. Samples of liver or other organs are then removed as rapidly as possible, and placed in covered vessels immersed in melting ice. The tissues

(mixed samples from four to six animals) are weighed cold, ground up quickly but quantitatively in small mortars with 6 per cent trichloroacetic acid solution, and the brei transferred to a measuring cylinder. Trichloroacetic acid is then added until the total volume in cc. is 8 times the number of gm. of tissue taken. The mixture is shaken and *filtered within 5 minutes*. For determination of free and total acid-soluble phosphorus 1 or 2 cc. aliquots are taken; the ester phosphorus is given by difference. The "free" phosphate is determined usually by the Bell-Doisy method, and the total acid-soluble phosphorus by a modification of the Briggs method.

*Phosphoric Esters of Red Blood Cells*—The oxalated blood, collected from four to six animals, is centrifuged, as soon as possible, for 15 minutes, the plasma removed, and some 4 cc. of the red blood cells pipetted from under the leucocyte layer with a wide mouthed pipette into a 10 cc. measuring cylinder. An approximately equal volume of 0.9 per cent NaCl solution is then added, and the contents of the cylinder thoroughly mixed. Duplicate samples are centrifuged at high speed in a hematocrit to determine the percentage volume of red blood cells in the mixture. 3 cc. samples of the mixture in duplicate are then pipetted into 22 cc. of water in a 50 cc. graduated cylinder, and the volume made up quickly to 30 cc. with 5 cc. of 25 per cent trichloroacetic acid. The cylinder is shaken for 10 seconds, kept for 5 minutes, and filtered. A 10 cc. sample of filtrate is taken for inorganic P, and 5 cc. samples for total acid-soluble P determinations. The assumptions are made (a) that all the organic acid-soluble P indicated by the difference between the free and the total acid-soluble figures comes from the red blood cells, and (b) that the hematocrit determination gives accurately the true volume of red blood cells.

A summary of experimental findings is given in Tables II and III.

#### DISCUSSION

From the findings in Tables II and III, the following conclusions might be drawn: (a) that the ester P per unit weight of liver tissue is definitely lowered in rickets; (b) that the addition of anti-rachitic agents to the otherwise rachitogenic diets increases the quantity of ester P in the liver towards the normal; (c) that the

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TABLE II

*Changes in Phosphoric Ester Content of Liver and Kidney in Experimental Rickets*

Experiment No.	Diet	x-Ray and bone ash findings	P compounds in tissue to nearest mg. P per 100 gm.		
			Inorganic P	Acid-soluble P	Ester P
Liver					
64	Normal	Normal	29	118	89
	McCollum Diet 3143	Rickets +++	34	99	<b>65</b>
	Same + cod liver oil	No rickets	27	105	78
	" + viosterol	" "	34	112	78
140	Normal	Normal	30	102	72
	High phosphorus	Osteoporosis	34	84	50
	Same + cod liver oil	Practically normal	30	100	70
	" + viosterol	" "	30	98	68
• 149	Normal	Normal	32, 30	97, 98	65, 68
	Steenbock Ration 2965 (A)	Rickets +++	30, 32	81, 80	<b>51, 48</b>
	Same + cod liver oil	Normal	28, 30	91, 93	63, 63
	Steenbock Ration 2965 (B)	Rickets +++	32, 33	74, 78	<b>42, 45</b>
	Same + cod liver oil	Normal	33, 30	86, 84	53, 54
Kidney					
64	Normal	Normal	26	97	71
	McCollum Diet 3143	Rickets +++	28	93	<b>65</b>
	Same + cod liver oil	No rickets	24	96	72
	" + viosterol	" "	28	98	70
140	Normal	Normal	26	106	80
	High phosphorus	Osteoporosis	30	91	<b>61</b>
	Same + cod liver oil	Practically normal	28	98	70
	" + viosterol	" "	29	106	77

Rachitic findings are given in bold-faced figures. It will be noticed that the inorganic phosphate of both liver and kidney does not seem to be lowered in rickets, in spite of the very low inorganic phosphate which characterizes the plasma when the disease is produced by the Steenbock or McCollum diet. The letters A and B following the diet number indicate minor modifications in the diet. +++ = severe; ++ = fairly severe.

ester P per unit volume of *red blood cells* is definitely lowered in rickets; (d) that this value is raised toward that of normal red

TABLE III

*Changes in Phosphoric Ester Content of Red Blood Cells in Experimental Rickets*

Experiment No.	Diet	Rickets (x-ray and bone ash findings)	P compounds in red blood cells to nearest mg. per 100 cc.		
			Inorganic P	Acid-soluble P	Ester P
149	Normal	Normal	8	66	58
	Steenbock Ration 2965 (A)	Rickets +++	4	48	<b>44</b>
	Same + cod liver oil	Normal	6	61	55
	Steenbock Ration 2965 (B)	Rickets +++	3	45	<b>42</b>
	Same + cod liver oil	Normal	6	62	56
154	Normal	"	9	79	70
	"	"	10	77	67
	0.5 per cent beryllium carbonate + normal diet	Rickets +++*	5	47	<b>42</b>
	" " "	" +++*	5	49	<b>44</b>
	2 per cent beryllium carbonate + normal diet	" +++*	4	37	<b>33</b>
155	Same + viosterol	" +++*	3	36	<b>33</b>
	Normal	Normal	7	78	71
	Steenbock Ration 2965 (C)	Rickets +++	3	37	<b>34</b>
	Same + cod liver oil	Very slight rickets	5	52	47
	Steenbock Ration 2965 (D)	Rickets +++	4	43	<b>39</b>
166	Same + cod liver oil	Normal	8	61	53
	Normal	"	7	75	68
	0.25 per cent beryllium carbonate + normal diet	Rickets ++*	4	57	<b>53</b>
	Steenbock Ration 2965	" +++	4	53	<b>49</b>
	Same + cod liver oil	Normal	5	61	56
	" + viosterol	"	6	69	63

Rachitic findings are given in bold-faced figures. Steenbock's rachitogenic Ration 2965 was used; the letters A, B, C, D, merely represent minor modifications in the diet, which have no significance here. +++ = severe; ++ = fairly severe.

\* "Beryllium" rickets (10).

blood cells by the addition of antirachitic agents to an otherwise rachitogenic diet.

Before these conclusions can be accepted, we must satisfy ourselves that certain fallacies have been avoided. Thus the fall in ester P may be due (1) to there being a larger percentage of water

in the rachitic liver tissue than in the normal, (2) to the effect of the shortage of phosphorus in the diet quite apart from there being any production of rickets, (3) to there being a normal variation in the proportion of phosphoric ester in the liver or red blood cells with size of animal, since in all the experiments the rachitic animals, though they usually grew well, were definitely smaller than the normals.

TABLE IV

*Variations in Phosphoric Ester Content of Liver and Red Blood Cells with Age and Size of Rat*

No. of animals	Average weight	Age	Normal or rachitic	Acid-soluble ester P	
				Per 100 gm. liver	Per 100 cc. red blood cells
	gm.	days		mg.	mg.
4	50	30 $\pm$ 2	Normal	56	79
6	61	30	"	58	78
4	90	40 $\pm$ 2	"	64	66
6	91	52	"	80	67
6	116	52	"	76	63
3	129	46	"	78	68
3	152	47	"	90	
6	152	52	"	88	62
3	47	46 $\pm$ 1	Rachitic	48	49
3	75	46 $\pm$ 1	"	62	
6	67	50	"		37
3	71	46 $\pm$ 2	"	65	53
3	87	46 $\pm$ 2	"	70	
6	81	48	"	50	44
6	79	48	"	44	42
6	88	54	"	56	40

With regard to (1), this point was repeatedly checked, and it was found that the water content of normal and rachitic liver tissue was approximately the same, the difference did not exceed 1 or 2 per cent. With regard to (2), it is to be remarked that a minute quantity of cod liver oil or viosterol added to the rachitogenic diet maintained the ester P much nearer to the normal, and also that apart from such addition, under the experimental conditions that were employed, rickets inevitably followed the giving of the rachitogenic diet. Moreover, the "rachitic" effect on the phos-

phoric ester content of liver tissue is shown equally well on the high phosphorus diet as on the Steenbock (8) or McCollum (9) low phosphorus diets, and does not therefore seem to be due immediately to phosphorus shortage. With regard to (3), which is a far more serious potential objection, a number of determinations were made on the livers and red blood cells of normal rats of different sizes with the same technique (Table IV).

It will be seen from Table IV that the acid-soluble phosphoric ester content of the liver slowly increases with increasing weight and possibly also with increasing age. After scrutiny of all our figures on this point we are of the opinion that the difference between the rachitic value and that for a normal animal of the same age and weight is significant, though the lower values obtained in normal animals of smaller weight make the true diminution in phosphoric ester in the liver in rickets less striking. However, by taking as controls for our rachitic animals not normal rats on a normal diet but *rats which have received the rachitogenic diet together with a prophylactic amount of vitamin D for the same length of time*, it appears from Tables II and V that the rachitic liver is characterized by a somewhat lower phosphoric ester content.

With regard to the red blood cell values, however, the situation is quite straightforward, since the smaller and younger the normal animal, the *greater* the amount of phosphoric ester per unit volume of red blood cells, while in the better grown as well as in the smaller rachitic animals the phosphoric ester content is markedly below any of the normal values. If the effect of differences in bodily size on the liver compounds appears to minimize the fall in the liver phosphoric esters in rickets, the effect of changes in bodily size on the phosphoric esters of the red blood cells renders even more striking the rachitic fall which we have observed in the ester content of the latter. Moreover, the phosphoric ester content of the rachitic rat red blood cells is definitely raised towards the normal value by adding minute amounts of antirachitic supplements to the diet, without any marked effect on the weight or rate of growth of the animal.

In Table V are summarized the results of an experiment on the effect of adding curative supplements to the diet of animals rendered rachitic by Steenbock's rachitogenic diet. Thirty rats of 22 to 24 days of age were placed on the Steenbock diet for 24

days. At the end of this time, six animals were killed, blood analyses and x-ray photographs indicating rickets in each. The remainder of the animals, presumably all rachitic at this stage, were divided into three groups; to one group cod liver oil was given in curative quantities, to another viosterol in curative quantities, while the third group was maintained on the unchanged Steenbock diet. After 12 days, all the animals were killed and the findings summarized in Table V were obtained.

These findings with regard to the phosphoric ester content of the red blood cells in experimental rickets are of interest in connection with the phosphatase theory of bone salt deposition in growing bone (11, 12). In experimental rickets the phosphatase content of

TABLE V

*Effect, on Ester P of Red Blood Cells and Liver, of Adding Vitamin D to an Otherwise Rachitogenic Diet*

Diet	x-Ray and blood inorganic P findings	P compounds to nearest mg. per 100 cc. red blood cells or 100 gm. fresh liver tissue			
		Red blood cells		Liver	
		Inor- ganic P	Ester P	Inor- ganic P	Ester P
Steenbock Ration 2965	Rickets +++	4	37	30	44
Same + cod liver oil	Healed rickets	5	58	29	58
“ + viosterol	“ “	4	46	29	54

+++ = severe.

the bone does not seem to be diminished, but slightly increased, so that a shortage of enzyme is not responsible for the faulty bone formation. As regards its probable functional activity in bone formation, the bone enzyme in rat rickets is now seen to suffer from a 2-fold handicap. In addition to the shortage of inorganic phosphate in rachitic blood, the results given in Table III show that it is also faced with a considerable shortage of the chief substrate for its activity in the circulating blood; namely, the phosphoric esters of the red blood cells. Whether this is also true for human rickets remains to be shown.

## SUMMARY

In experimental rickets in rats the acid-soluble phosphoric ester content of unit volume of red blood cells is markedly diminished below the normal values for control animals on a normal diet, or the values for animals receiving the rachitogenic diet together with small quantities of vitamin D. The addition of therapeutic agents to the rachitic diets causes an increase toward normal in the quantity of phosphoric ester in the red blood cells.

A similar though less marked change appears to occur in liver tissue. In the case of the red blood cells the change is clear cut, but the situation with regard to the liver is complicated by a possible fallacy, discussed in the text, concerned with the size of the animals.

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## THE *o*-BENZOQUINONE TEST FOR CYSTEINE

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Dyer and Baudisch (1) recently reported a reaction of cysteine with *o*-benzoquinone which seemed to have a high degree of specificity comparable to the Sullivan (2) specific reaction for cysteine. The Sullivan reaction makes use of an *o*-quinone, 1,2-naphthoquinone-4-sodium sulfonate. The great capacity of 1,2-naphthoquinone-4-sodium sulfonate to react with other compounds with the production of complexes of high tinctorial power was early recognized by Witt and Kaufmann (3) who made the compound and by Böniger (4) who gave a number of its reactions of the oxyindophenol type. Ehrlich and Herter (5) and especially Herter (6) greatly extended the number of color reactions obtainable with 1,2-naphthoquinone monosodium sulfonate and indicated many biological applications of the reaction. Folin (7) employed the compound in his colorimetric method for the determination of the amino acids in blood. Sullivan's reaction differs from all other experiments with the 1,2-naphthoquinone-4-sodium sulfonate in that the color development occurs in a strongly reducing atmosphere. Cysteine, 1 mg. in 5 cc. of 0.1 N hydrochloric acid gives a striking red color in the Sullivan procedure while all other compounds tested, at least 150, give a light yellow. Among the compounds tested are amino acids, amines, thio compounds, and a long list of substances which may occur in plant or animal tissue or were mentioned by Herter as reacting in some way with 1,2-naphthoquinone-4-sodium sulfonate. The Sullivan reaction has a remarkable specificity. It has been used for complexes which yield cysteine by reduction, by hydrolysis, or by a combination of these processes. For a positive outcome as shown by Sullivan and Hess (8) it requires that the three groups of cysteine,—SH,

—NH<sub>2</sub>, —COOH, be free and in the order as occurring in natural cysteine since isocysteine is negative.

Dyer and Baudisch found that of the compounds tested by them only cysteine reacted with o-benzoquinone to give a red color in the chloroform layer and considered that their test was specific qualitatively for cysteine. The Dyer and Baudisch criterion is that when an aqueous solution of cysteine hydrochloride is shaken with a chloroform solution of o-benzoquinone a deep red color is produced in the chloroform layer, a phenomenon that did not occur with any of the other amino acids and sulfur-containing

TABLE I

*Reaction of Various Compounds in o-Benzoquinone Test for Cysteine, Results for Which Were All Positive*

Substance, concentration 1 mg. per cc.	Chloroform layer	Substance, concentration 1 mg. per cc.	Chloro- form layer
Cysteine.....	Red	Isoamylamine.....	Red
Benzidine.....	"	n-Propylamine.....	
Aniline.....	Strong red	n-Butylamine.....	
Anthranilic acid.....	Good red	Disulfide of dithiotyro-	
Indole in 40 per cent	" "	sine (after reduction)	
alcohol.....		Chloroacetyl cystine	
Cystine amine HCl ( $\beta,\beta'$ -		(after reduction).....	
diaminodiethyl-disulfide		Trimethylamine.....	
dihydrochloride) after		Acetonitrile.....	
reduction.....	Red		
Aminoguanidine car-			
bonate.....			

compounds tested by them. We verified their findings with a positive cysteine reaction and a negative reaction with other amino acids and various sulfur compounds. Their testing, however, did not go far enough as proved by Table I which shows that a number of compounds give a red chloroform layer in the procedure of Dyer and Baudisch. In the Sullivan method these compounds are all negative.

The fact that the compounds given in Table I react more or less like cysteine shows that the Dyer and Baudisch test may hardly be regarded as a highly specific reaction for cysteine. This conclusion does not detract from the finding that the Dyer and Bau-

disch reaction with *o*-benzoquinone differentiates cysteine from other amino acids and from cystine, glutathione, and a number of sulfur compounds. The reaction, however, is considerably interfered with by the presence of glutathione. Dyer and Baudisch report this interference but imply that it is readily overcome by adding more of the quinone. With increasing ratios of glutathione to cysteine, however, we found the cysteine reaction with benzoquinone difficult to get. With a mixture of 10 mg. of reduced glutathione and 1 mg. of cysteine per cc. the reaction with *o*-benzoquinone is absolutely negative even with large increases of reagent and long standing. In such a mixture as shown by Sullivan and Hess (9) cysteine can be estimated quantitatively by the Sullivan procedure with 1,2-naphthoquinone-4-sodium sulfonate.

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# THE EFFECT OF ANTICOAGULANTS ON DETERMINATIONS OF INORGANIC PHOSPHATE AND PROTEIN IN PLASMA

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Inorganic phosphate estimations are commonly carried out on serum from clotted blood, or on plasma from blood containing anticoagulants. Available data leave one in doubt as to whether the inorganic phosphate content in the two cases is identical and agrees with that of so called native plasma; that is, plasma obtained by chilling blood or collecting it in paraffined tubes and centrifuging before clotting occurs.

Addition of oxalate to whole blood does not alter its inorganic phosphate content (1) and in this respect is preferable to defibrination, which is accompanied by a change of inorganic to organic phosphate (2). Moreover, Tolstoi (3) found good agreement between inorganic phosphate values for oxalate plasma and serum of human blood, the serum being separated after 3 hours. Likewise Buell (4) found agreement between the inorganic phosphate values for citrate plasma and native plasma of dog blood, and Wang and Felsher (5) found that citrate plasma and serum of sheep blood contained the same amount of inorganic phosphate. On the other hand, Buell found that oxalate plasma contained less phosphate than native plasma of dog blood. It is also known that in cell volume determinations hypertonic anticoagulants shrink the cells and dilute the plasma. Further evidence of this dilution is found in a fall in the serum proteins of defibrinated blood (6) on addition of 0.2 per cent of potassium oxalate. Finally, Barrenscheen, Doleschall, and Popper (1) found no agreement between inorganic phosphate values for native plasma and those for serum obtained from the same human blood samples within 2 hours. In eleven cases plasma gave the lower value six times, and the higher value

five times, the differences being evenly distributed between +0.92 and -0.94 mg. of inorganic phosphorus per 100 cc. If variations of this size occur, agreement between serum and anticoagulant plasma might simply lay both values open to question.

The inorganic phosphate determination is used clinically in two connections in which small variations are important; namely, in determination of the inorganic phosphate curve after glucose administration, and in determination of calcium and phosphate products. In the former instance the fall in phosphate is small (7), and in the latter any error in the phosphate value is multiplied by the calcium value. The writer has therefore studied some of the variables involved in the estimation of phosphate (such as the effect of sodium fluoride, sodium citrate, potassium oxalate, or heparin), possible changes in the phosphate content of oxalate plasma during 5 hours, and differences between native plasma and oxalate plasma or native plasma and serum. A comparison was also made between the methods of Benedict and Theis (8) and Fiske and Subbarow (9).

#### *Determination of Phosphate in Presence of Fluoride*

In contrast with oxalate and citrate, fluoride interferes slightly with the color reaction used in the above methods for estimating phosphate when it is used in the usually recommended amount of 10 mg. per cc. of blood. In the presence of 4 times this amount of fluoride no color reaction whatever is obtained in one of the methods and very little in the other. This effect is easily overcome by addition of aluminum chloride.

In Table I are recorded the results of an experiment with variable amounts of aluminum chloride and constant amounts of fluoride. The two compounds were added as solutions, replacing a part of the water required when the indicated amounts of phosphorus are determined in a total volume of 10 cc. The method of Fiske and Subbarow is more sensitive to fluoride interference than the other method, and therefore yields the more striking result. In Tube 4 (Table I) the interference with color development is still practically complete, while in Tube 5 it has been almost completely prevented. In the latter tube the ratio of aluminum to fluorine is the same as in the compound  $AlF_3$ , hence the probable explanation of the above result is the formation of weakly ionized

aluminum fluoride. Comparison of Tubes 7 and 8 (Table I) shows that addition of aluminum chloride introduces no blank in either analytical method, while sodium fluoride introduces a small blank in the Benedict-Theis method only. With smaller amounts of fluoride this becomes negligible, but some c. p. grades of sodium fluoride contain the substance responsible in amounts making them unfit for use. Experiments with fluoride added to blood serum gave results comparable with those above—complete interference with large amounts of fluoride, and complete prevention of this interference by aluminum chloride. Adding to the 5 cc. of trichloroacetic acid filtrate 1 cc. of a 20 per cent solution of

TABLE I

*Showing That Aluminum Chloride Prevents Interference of Fluoride in Colorimetric Determinations of Phosphate*

Tube No.	NaF	AlCl <sub>3</sub> 6H <sub>2</sub> O	Benedict-Theis method		Fiske-Subbarow method	
			P taken	P found	P taken	P found
	mg.	mg.	mg.	mg.	mg.	mg.
1	0	0	0.025	0.025	0.040	0.040
2	42	0	0.025	0.010	0.040	0.000
3	42	40	0.025	0.019	0.040	0.000
4	42	60	0.025	0.022	0.040	Trace
5	42	80	0.025	0.025	0.040	0.036
6	42	161	0.025	0.026	0.040	0.040
7	42	241	0.025	0.026	0.040	0.040
8	0	241	0.025	0.025	0.040	0.040

aluminum chloride (AlCl<sub>3</sub>·6H<sub>2</sub>O) in place of an equal amount of water is thus a simple means of preventing any interference due to fluoride.

*Comparison of Methods, and of Anticoagulant Plasma and Serum Values*

In Table II are shown the analytical results for anticoagulant plasma and serum of dog blood and human blood. 30 cc. samples of blood were drawn and discharged into two tubes, one of which contained an accurately measured portion of a concentrated solution of anticoagulant not greater than 1 per cent of the volume of blood added. Sodium fluoride was added as a solid. The solu-

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tions were used on the assumption that the smaller amount of agitation required would minimize loss of  $\text{CO}_2$  and changes in inorganic phosphate, but parallel tests carried out later indicate that results with solid anticoagulants are the same. The following anticoagulants were used: sodium fluoride, 10 mg. per cc. of blood; potassium oxalate, 2 mg. per cc.; sodium citrate, 4 mg. per cc.; and heparin, 0.4 mg. per cc. Sodium fluoride caused some hemolysis. The amount of the other anticoagulants is well below that which

TABLE II  
*Comparison of Serum with Plasma Containing Various Anticoagulants*

Inorganic P per 100 cc				Inorganic P of serum Inorganic P of plasma	Total N of serum Total N of plasma	Anticoagulant	Source of blood
Serum		Plasma					
Benedict- Thies method	Fiske- Sub- barow method	Bene- dict- Thies method	Fiske- Sub- barow method				
mg	mg	mg	mg				
6 50	6 50	5 63	5 55	1 16	1 15	Na fluoride	Dog
4 33	4 25	3 50	3 38	1 25	1 32	" "	"
5 48	5 30	4 30	4 35	1 25	1 34	" "	"
4 00	4 00	3 40	3 37	1 18	1 09	K oxalate	"
5 05	5 00	4 58	4 53	1 10	1 03	" "	"
3 34	3 22	2 90	2 82	1 15	1 05	Na citrate	"
4 72	4 60	4 08	3 91	1 17	1 07	" "	"
4 18	4 07	3 94	3 82	1 06	0 99	Heparin	"
3 37	3 26	3 30	3 12	1 03	1 02	"	"
3 48	3 34	2 76	2 94	1 20	1 21	Na fluoride	Human
3.78	3 81	3 20	3 36	1 16	1 29	" "	"
3 34	3 31	2 92	2 97	1 13	1 06	K oxalate	"
3 20	3 16	2 76	2 69	1 17	1 03	" "	"
3 68	3 61	3 24	3 16	1 14	1 03	" "	"

will cause hemolysis, or interference with the color reaction used in determining phosphate.

The tubes containing blood with and without anticoagulant stood side by side at room temperatures near  $25^\circ$  for 30 minutes, this being about the minimum time for separation of serum. They were then centrifuged for 5 to 10 minutes. Analyses by each method were carried out in duplicate. An examination of the results shows that there is good agreement between the two analytical methods, whether they are applied to serum or plasma

of normal dog blood or normal human blood having a fairly wide range of phosphate content. It is also apparent that the values for anticoagulant plasma are always decidedly lower than those for serum of the same blood sample. In the case of dog blood the difference is largest when sodium fluoride is used; potassium oxalate and sodium citrate in the amounts required have a smaller effect, and heparin lowers the plasma value very little. Unfortunately, heparin preparations contain material which is transmitted to trichloroacetic acid filtrates and causes slight turbidity in the determination. When the turbidity is removed by centrifuging there still remains a small blank which differs for the two analytical methods, probably because heparin preparations contain organic phosphate (10). Heparin is thus an undesirable anticoagulant in this connection. The few results which are recorded have had a blank of 0.2 to 0.3 mg. of inorganic phosphorus per 100 cc. of plasma subtracted.

The sixth column of Table II shows the ratio of serum total nitrogen to plasma total nitrogen, both determined by the method of Howe (11). Although serum does not contain fibrinogen, it always contains more protein than anticoagulant plasma, except when heparin is used. The small amount of nitrogen in the heparin is entirely negligible in this connection; the fibrinogen nitrogen, if added to that of the serum, would increase all of the ratios recorded by an average of 0.06. A comparison of the protein content of oxalate plasma and native plasma is recorded in the following section.

#### *Comparison of Serum and Oxalate Plasma with Native Plasma*

In the following experiments an attempt was made to determine whether the difference found between serum and anticoagulant plasma is due entirely to changes produced by the anticoagulant, such as dilution of plasma by dehydration of the corpuscles, and altered distribution of inorganic phosphate between cells and plasma, or whether the serum values are also open to question. Serum and oxalate plasma were therefore compared with native plasma, obtained about 6 minutes after venepuncture, low temperature being used to delay coagulation.<sup>1</sup> Serum was separated

<sup>1</sup> The following technique was used. 15 cc. conical centrifuge tubes were stoppered and placed in trunnions intended for 50 cc. tubes. The tubes

after 30 minutes standing at room temperature, as in the preceding experiments, while oxalate plasma was separated after the same length of time, but with the difference that the specimen was placed in the refrigerator during this interval.

The results are given in Table III. With a single exception, serum separated after 30 minutes contained more inorganic phosphate than native plasma of the same specimen, and oxalate plasma contained less than native plasma in every case. The difference between native plasma and serum did not vary as widely as in the

TABLE III  
*Comparison of Serum and Oxalate Plasma with Native Plasma*

Inorganic P per 100 cc						Source of blood
Native plasma	Serum	Difference	Native plasma	Oxalate plasma	Difference	
<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	
3 33	3 52	+0 19	4 60	4 30	-0 30	Dog
5 48	5 67	+0 19	3 73	3 51	-0 22	"
3 22	3 40	+0 18	3 37	3 07	-0 30	"
3 49	3 40	-0 09	3 30	2 98	-0 32	Human
3 33	3 49	+0 16	3 05	2 81	-0 24	"
3 84	4 01	+0 17	2 96	2 70	-0 26	"
3 03	3 50	+0 47	3 02	2 86	-0 16	"
3 44	3 70	+0 26	3 62	3 35	-0 27	"

The above analyses were carried out on two series of bloods, one series serving for comparison of native plasma and serum, the other for comparison of native plasma and oxalate plasma

studies of Barrenscheen *et al.* (1), to which reference was made above, but in the present limited series of human bloods it varied more than the difference between oxalate plasma and native plasma.

were held in the center of the trunnions by rubber bands passing over the stopper and around the lower end of the trunnion. The space between tube and trunnion was filled with water, and the outfit was then placed in a freezing mixture until the water surrounding the tube had frozen. Part of the blood drawn was discharged directly into such tubes and centrifuged for 5 minutes. Samples of plasma were then withdrawn and analyzed at once. The remainder of the blood drawn was allowed to clot, or was mixed with oxalate equivalent to 0.2 per cent final concentration.

Total nitrogen determinations were carried out on the oxalate plasma and native plasma of the last three blood samples in Table III. The nitrogen in oxalate plasma was 92.3 to 96.5 per cent of that in native plasma. On an average, the oxalate plasmas contained 5.0 per cent less nitrogen, and 7.2 per cent less inorganic phosphorus than the native plasmas.

*Stability of Oxalate Plasma at Low Temperature*

While the initial change in plasma composition which is caused by oxalate is a disadvantage, oxalate plasma also has a point in its

TABLE IV  
*Constancy of Inorganic Phosphorus Content of Oxalate Plasma in Contact with Corpuscles at 10°*

Specimen No.	Inorganic P in 100 cc. oxalate plasma Min. after venepuncture			
	20	60	120	300
	mg.	mg.	mg.	mg.
1	2.06	2.12	2.03	2.09
2	2.14	2.07	2.10	2.14
3	3.16	3.20	3.20	3.20
4	3.65	3.55	3.48	3.55
5	2.72	2.72	2.80	2.76
6	2.34	2.30	2.34	2.37
7	2.61	2.54	2.57	2.55
8	2.28	2.32	2.29	2.27
9	2.56	2.54	2.54	2.62
10	2.65	2.62	2.69	2.62

favor; namely, that its inorganic phosphate content remains unchanged for at least 5 hours when the oxalated blood is kept in the refrigerator at the usual temperatures of 8–10°. This may be partly accounted for by the fact that both cold and oxalate inhibit glycolysis, with its attendant changes of acidity. The results in Table IV were obtained as follows: Freshly drawn samples of human blood were oxalated and divided among four tubes, one of which was centrifuged within 20 minutes, while the others were placed in the refrigerator and centrifuged after 1, 2, and 5 hours, respectively. The inorganic phosphate content of the plasma remained almost constant. This is important, since in determina-

tion of the phosphate curve following glucose administration the blood samples are drawn over a period of  $2\frac{1}{2}$  hours, and unless each sample is analyzed separately, the specimens will stand a variable length of time before analysis.

*Effect of Varying the Amount of Oxalate*

Six samples of human blood were drawn, and each was discharged into two tubes containing potassium oxalate sufficient to make a final concentration of 0.2 per cent and 0.4 per cent respectively. The average inorganic phosphorus content of the plasma from blood with 0.4 per cent of oxalate was 0.16 mg. per 100 cc. below that containing 0.2 per cent. The quantity of oxalate should be controlled by measuring the amount of solution placed or evaporated in the tubes, and by adding a uniform amount of blood from the syringe.

DISCUSSION

Since the serum and oxalate plasma separated 30 minutes after the blood is drawn yield two different inorganic phosphate values, neither of which is identical with that of native plasma, it is in a sense unfortunate that serum and anticoagulant plasma have been used almost to the exclusion of whole blood in clinical studies involving the determination of phosphate. Determinations on whole blood are unaffected by an altered distribution of phosphate between cells and plasma, or by changes in plasma volume as the result of using anticoagulants. The precipitation and determination must, however, be carried out at once, for neither the whole blood nor the acid filtrate of it is stable as to its inorganic phosphate content (2, 10). The writer has observed that in trichloroacetic acid filtrates of human corpuscles the inorganic phosphate, calculated as phosphorus, per 100 cc. of corpuscles, increased 0.26 to 0.49 mg. in 5 hours. The stability of plasma filtrates, because of their negligible organic phosphate content, and the stability of oxalate plasma when kept in the refrigerator, even though it remains in contact with the cells, avoid the necessity of carrying out each determination separately. In comparative studies it should, however, be borne in mind that the phosphate content of oxalate plasma is definitely lower than that of serum or native plasma.

The effect of fluoride in the amount usually used to inhibit gly-

colysis is obviously too great to permit its use when plasma is to be obtained, even though its direct interference with the color reaction for phosphate is easily overcome. The possibility of stabilizing the inorganic phosphate content of whole blood by means of fluoride has been partially investigated, since the writer finds that the increase of inorganic phosphate in laked defibrinated blood is decidedly less marked in the presence of fluoride. The changes in whole blood are, however, not completely inhibited by fluoride alone.

#### CONCLUSIONS

1. Fluoride interferes with the color reaction used in the Benedict-Theis and Fiske-Subbarow methods for determination of inorganic phosphate. The interference is prevented by aluminum chloride.

2. Plasma obtained in 30 minutes, with fluoride, citrate, or oxalate as anticoagulants, always contains less inorganic phosphate than serum, and also less total nitrogen, despite the presence of fibrinogen. Dilution of the plasma with water drawn from the cells would seem to account for most of the fall in phosphate.

3. When native plasma is used as the standard, the inorganic phosphate content of oxalate plasma of human blood and dog blood is too low, and that of serum is too high.

4. The inorganic phosphate content of oxalate plasma left in contact with the cells at 10° remains unchanged for 5 hours.

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# THE FATE OF THE ANTIRACHITIC FACTOR IN THE CHICKEN

## II. THE EFFECTIVENESS OF THE FACTOR ADMINISTERED BY MOUTH AND INTRAPERITONEALLY\*

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The non-equivalence of the antirachitic potency of cod liver oil and irradiated ergosterol in the prevention of leg weakness in the growing chick has been noted by several investigators. Reference has been made to this work and to the results obtained in this laboratory in earlier papers (1, 2).

In a series of studies of the fate of the antirachitic factor in the growing chick it was of interest to determine whether destruction of the factor took place in the digestive tract. In an earlier study (2) it was found that only 26.5 per cent of the rat units of antirachitic factor in the case of ergosterol-fed chicks and only 34.1 per cent in the case of those receiving cod liver oil could be recovered in the droppings. The remainder could not be accounted for and the question as to whether partial destruction took place in the digestive tract was, of course, in order. The present investigation is an attempt to answer this question.

The experimental plan called for a comparison of the antirachitic potency of the two sources of the factor when administered by capsule and by intraperitoneal injection. Bone ash percentage and the calcium and inorganic phosphorus of the blood were used as criteria of the comparative effectiveness of the two methods of administration.

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Although no reports of injection studies with chicks have come to the attention of the authors, studies with other species have been made. Zucker and Matzner (3) have reported that the subcutaneous injection of the active principle of cod liver oil caused no healing of rickets in rats but other investigators have found both vitamins A and D to be effective when administered parenterally. Thus, vitamin A given parenterally has been noted by Wollman and Vagliano (4) to have practically the same effect on growth as when fed. Blegvad (5) reported the successful treatment of a clinical case of xerophthalmia by subcutaneous injection of a vitamin A concentrate. In the hands of Soames (6) the intraperitoneal injection of cod liver oil brought about improved growth in rats on a diet deficient in vitamin A and on a rickets-producing diet improved calcification was observed. Hume, Lucas, and Smith (7) have reported that the antirachitic factor in irradiated cholesterol can be absorbed from the skin of rats and rabbits in an amount sufficient to supply the needs of the animal. According to Wilkins and Kramer (8) the intramuscular injection of a cod liver oil concentrate gave satisfactory results in two children suffering from active rickets. In an extensive study of the utilization of fatty oils given parenterally, Koehne and Mendel (9) observed that "both vitamins A and D in cod liver oil can be utilized by young rats when the oil is administered parenterally."

In an investigation by Reed and Thacker (10) the objective was essentially the same as that of the present report; namely, to determine whether irradiated ergosterol is more effective as an antirachitic agent when administered by some route other than by mouth. They found that intravenous or intraperitoneal injection was more effective in the dog than comparable doses fed by mouth.

#### EXPERIMENTAL

250 newly hatched white Wyandotte chicks were distributed in seven lots, so that each represented the weight distribution in the original group. An all-mash basal ration, devoid of the antirachitic factor, was supplied to all groups and had the following composition: ground yellow corn 47 per cent, wheat middlings 20 per cent, wheat bran 15 per cent, dried buttermilk 5 per cent, meat

scrap 5 per cent, fish meal 4 per cent, finely ground oyster shell 3 per cent, and sodium chloride 1 per cent. The calcium content of the ration was 1.88 per cent and the phosphorus 0.97 per cent, the Ca:P ratio being 1.94.

Cod liver oil was administered at the 1 per cent level and irradiated ergosterol solution in an equal volume but of an antirachitic potency 6 times that of the cod liver oil. The weekly consumption of cod liver oil at the 1 per cent level was calculated from existing data (11) on food consumption by chicks of another heavy breed, namely Rhode Island Reds. The volume of oil used and the number of rat units administered during a week are displayed in Table I.

TABLE I  
*Feeding Schedule of Antirachitic Factor*

Wk.	Volume of oil used	Rat units furnished by		No. of capsule feedings per wk.	No. of injections per wk.	Volume of oil per injection
		Cod liver oil	Irradiated ergosterol			
	cc.					cc.
1	0.50	10.3	61.8	5	2	0.25
2	0.95	19.6	117.4	5	2	0.47
3	1.35	27.8	166.9	6	2	0.67
4	1.90	39.1	234.8	6	2	0.95
5	2.60	53.6	321.4	6	2	1.30
6	3.25	67.0	401.7	6	2	1.62
7	3.60	74.2	445.0	5	2	1.80

The experimental treatment of the lots was according to the following scheme.

- Lot 1, cod liver oil by capsule
- " 2, " " " intraperitoneally
- " 3, irradiated ergosterol solution by capsule
- " 4, " " " intraperitoneally
- " 5, corn oil (same volume as Lot 4) by capsule
- " 6, " " " " " 4 intraperitoneally
- " 7, basal ration only

For the determination of antirachitic potency the Steenbock technique, as outlined by the Wisconsin Alumni Research Foundation, was employed. The cod liver oil contained 21 rat units per cc. and the irradiated ergosterol solution 1250 rat units per cc. of

0.1 per cent stock solution in corn oil. The latter was diluted so that it had a potency 6 times that of an equal volume of cod liver oil.

The possibility of leakage after injection, and ingestion of the oil from the feathers, was recognized and an examination of the site of injection made in each case. In earlier trials leakage was noted after subcutaneous injections, but in the present experiment care was taken that the needle actually passed through the peritoneum and with this technique there was no leakage. The abdominal wall was lifted and the needle directed in a manner to avoid puncture of any internal organs. The oils were not sterilized but there was no postmortem evidence of any infection. The natural resistance of the chicken presumably served as a protection.

At the end of the 7th week of the experiment, fifteen birds from each lot were selected at random to be used for blood analyses and bone ash determinations. After samples of heart blood had been taken the chicks were killed by decapitation and the left tibias removed. Bone ash determinations were made on the left tibias of the individual birds of each lot as described in a previous paper from this laboratory (12). Blood calcium was determined by the Tisdall (13) modification of the Kramer and Tisdall method and inorganic phosphorus by the Briggs (14) modification of the Bell-Doisy method. Individual birds were used for the blood studies.

#### DISCUSSION

Table II gives in summary the mean values for bone ash, serum calcium, and inorganic phosphorus for all of the groups. A statistical analysis indicates that the differences of 3.48 per cent in mean bone ash values between Lots 1 and 3 and of 4.89 per cent between Lots 2 and 4 are significant. Similarly the differences of 4.76 per cent between Lots 3 and 7 and of 3.42 per cent between Lots 4 and 7 are of significance. The chicks of Lots 3 and 4 were not as fully protected against leg weakness by the amount of irradiated ergosterol supplied, according to the bone ash percentages, as were those of Lots 1 and 2 which received cod liver oil. In general appearance the chicks of Lot 3, ergosterol-fed, were the equal of the lot fed cod liver oil, although the average weight was less than that of the latter (Table III).

A consideration of the mean bone ash values of Lots 1 and 2, cod liver oil-fed and injected, shows that there is no significant difference between the lots, indicating that the chick can assimilate the antirachitic factor from cod liver oil when injected intra-

TABLE II  
*Summary of Average Bone Ash Percentages and Calcium and Inorganic Phosphorus Contents of Blood Serum*

Group No.	No. of determinations	Average bone ash and probable error	Coefficient of variation	Average Ca per 100 cc serum and probable error	Coefficient of variation	Average P per 100 cc serum and probable error	Coefficient of variation
		per cent	per cent	mg.	per cent	mg.	per cent
1. Cod liver oil-fed...	15	47.93 $\pm$ 0.217	2.6	9.8 $\pm$ 0.133	7.8	8.1 $\pm$ 0.213	15.1
2. Cod liver oil-injected...	15	48.00 $\pm$ 0.452	5.4	9.6 $\pm$ 0.215	12.9	6.4 $\pm$ 0.223 (14)*	13.2
3. Irradiated ergosterol-fed...	15	44.45 $\pm$ 0.457	5.9	9.4 $\pm$ 0.162	9.9	8.0 $\pm$ 0.210	15.1
4. Irradiated ergosterol-injected....	16	43.11 $\pm$ 0.488	6.7	8.8 $\pm$ 0.210	14.2	6.0 $\pm$ 0.263 (13)	23.4
5. Corn oil-fed.....	14	40.07 $\pm$ 0.442	6.1	7.3 $\pm$ 0.258 (13)	18.9	8.3 $\pm$ 0.424 (12)	26.2
6. Corn oil-injected..	15	41.40 $\pm$ 0.443	6.1	7.7 $\pm$ 0.260	19.6	5.6 $\pm$ 0.202 (14)	20.0
7. Basal.....	16	39.69 $\pm$ 0.341	5.2	6.7 $\pm$ 0.192 (17)	17.6	6.2 $\pm$ 0.231	22.3

\* Numbers in parentheses indicate a number of determinations different from that shown in the second column.

peritoneally. In the case of cod liver oil the amount used was probably more than enough to meet the minimum requirements and a small difference in effectiveness of the two modes of administration might not manifest itself. The amount of irradiated

ergosterol used was not sufficient to meet the needs of the birds and any difference in effectiveness of the two methods should become apparent. Although the lower bone ash percentage of the injected group suggests that intraperitoneal injection is less effective than feeding, from the statistical standpoint the difference between bone ash percentages is not significant. Hence the effectiveness of the antirachitic factor when injected was essentially the same as when fed by capsule. It appears therefore, that if there is a decrease in effectiveness of the antirachitic factor in the form of irradiated ergosterol in the body of the chick it does not take place in the digestive tract to a greater extent than in the body tissues. It should be recalled that Reed and Thacker (10) found intravenous or intraperitoneal injection to be more effective in the dog than comparable doses fed by mouth.

There are not significant differences among the mean bone ash values for Lots 5, 6, and 7, which demonstrates that the corn oil which was used for dilution of the irradiated ergosterol had no antirachitic potency either when injected or fed by capsule. The value for Lot 6, corn oil-injected, however, is slightly higher than those of Lots 5 and 7 and is not markedly different from that of Lot 4, irradiated ergosterol-injected, in which only partial protection against leg weakness was produced.

A statistical consideration of the mean serum calcium values shows no significant differences between the lots receiving cod liver oil, fed and injected, or the lots receiving irradiated ergosterol, administered in the same manner. The four lots which received the antirachitic factor show calcium concentrations significantly higher than those of the lots which were deprived of it, although in the case of Lots 4 and 6 the difference is not as pronounced as in the other instances. The values for Lots 2 and 3 are essentially the same as those for Lot 1, which is considered to be normal for this experimental régime, while the difference between Lots 1 and 4 is sufficient to have a slight degree of significance statistically. As in the case of the bone ash values, the calcium value of Lot 4 is less than that of Lot 3 and suggests that the injection of irradiated ergosterol was not as effective as feeding by capsule. Statistically, however, the difference is not significant. The marked difference between the lots which received cod liver oil and those to which irradiated ergosterol was administered, which

was shown by the bone ash values, is not apparent in the case of the calcium of the blood serum. It is of interest that the capsule feeding of irradiated ergosterol maintained a calcium and inorganic phosphorus level of the blood serum essentially the same as that in the case of capsule feeding of cod liver oil, yet the bone ash percentage in the latter case was significantly higher than in the former.

The average values for inorganic phosphorus of the blood serum of the capsule-fed groups are significantly higher than for the corresponding injected groups, Table II, even in the case of those receiving corn oil. Furthermore, they are higher than those that have been obtained previously in this laboratory (1) with chicks of the same age and raised under comparable conditions but which have consumed the factor admixed with the ration. The serum calcium, on the other hand, shows no significant variation between the groups which received the factor by the two methods of administration. The phosphorus values for the injected group are more nearly like those obtained when the factor is mixed with the ration. The value for the basal group is of the order usually encountered with a ration of this type.

To determine whether errors were being made in the use of the analytical method it was examined critically. The phosphate solution used in the preparation of the color standard was carefully checked by gravimetric analysis and found to contain the required amount of phosphorus. The serum was separated within 3 hours and the analyses completed within 12 hours after the samples were drawn. All lots were handled in the same manner, so that even if a constant error were present the fact would remain that a higher value was obtained in the capsule-fed groups.

In previous experiments, in which lower blood phosphorus values were found, a pooled blood sample from a group was obtained from the blood vessels severed by decapitation, but by this method it was not possible to collect the same volume of blood from each bird. To determine whether the method of bleeding affected the phosphorus level, blood was collected from ten birds each of Lots 1 and 3 by the decapitation method. Analysis of the serum from the pooled blood of these lots gave values which were essentially the same as those obtained when bleeding from the heart was practised. Hence, the method of bleeding was not a fac-

tor in causing an inorganic blood phosphorus level higher than usual. At the present time no data are available on which to base an explanation of the high phosphorus values. Several tentative suggestions might be made, among them being one that a phosphorus-raising principle is present, which is protected from destruction during contact with the food before ingestion or while in the fore part of the digestive tract. Hess and associates (15) have reported normal concentrations of inorganic phosphorus unaccompanied by healing of the rachitic lesion in the rat. However, when Kramer, Shear, and Siegel (16) repeated experiments of the type reported by Hess and coworkers, they found that "whenever

TABLE III  
*Distribution of Chicks and Weekly Weights during Experiment\**

Lot No	Initial No of chicks	Initial average weight	Average weight							No of chicks at end of experiment (7 wks)
			1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk †	
		gm	gm	gm	gm	gm	gm	gm	gm	
1	52	46 0	79 2	133 0	200 6	255 6	337 2	437 0	557 0	49
2	25	48 0	69 0	106 0	174 0	232 0	280 5	351 0	410 5	20
3	53	49 2	77 6	125 0	188 0	243 8	321 3	390 2	474 0	48
4	24	48 2	79 8	121 0	181 0	231 5	288 5	354 4	373 0	17
5	20	47 6	79 2	127 4	187 0	227 8	280 0	316 3	371 5	14
6	24	50 2	72 7	117 1	174 0	224 5	269 0	333 0	393 0	18
7	63	47 0	71 2	115 0	180 0	225 0	276 6	304 8	337 0	46

\* The number of chicks in Lots 1, 3, and 7 was larger than in the other lots in order to furnish bile for a subsequent experiment.

† 7.5 weeks of age.

the Ca  $\times$  P product was raised above the ricketic level, healing was noted." In the present study the inorganic phosphorus value for the corn oil-fed group was as high as that of the cod liver oil- and ergosterol-fed groups, and significantly higher than that of the control group, yet the bone ash percentage indicates the leg weakness condition quite definitely. In this instance, however, the calcium level of the blood was considerably lower than that of the non-rachitic groups.

The weekly weights of the seven lots are shown in Table III. It will be noted that the lot injected with cod liver oil and that injected with irradiated ergosterol in corn oil did not show nearly

as good growth as the corresponding lots which were fed the supplements by capsule. An autopsy of the chicks at the time they were killed revealed that the internal organs were literally bathed in unabsorbed oil within the peritoneal cavity. In the case of Lot 2, injected with cod liver oil, there was an accumulation of yellow, slimy, partially solidified material in the abdominal region. It appeared to be a fibrinous exudate sometimes flaky or granular in character. In some instances the exudate formed a membrane which adhered firmly to the capsule of the liver and the abdominal air sacs. In several of the chicks in this lot the oily material had accumulated to such an extent in the lower abdominal region, that a considerable amount of pressure was apparently put upon the organs which it surrounded. In the lots in which corn oil was the injected material, Lots 4 and 6, several cc. of oil were pipetted from the abdominal cavity, but the exudate was not observed. Koehne and Mendel (9) in their work with rats, a portion of which was devoted to the study of the utilization of vitamins A and D from cod liver oil administered intraperitoneally, found that the oil so given had toxic properties as evidenced by the marked deterioration in physical condition of all the animals used. It seems probable that the inferior growth shown by the chicks of the injected lots as compared with the lots fed the same supplement is a result of the accumulation of unabsorbed oil within the peritoneal cavity.

#### SUMMARY

1. Essentially the same bone ash percentages were obtained by administering the antirachitic factor by injection as by capsule, either in the form of cod liver oil or of irradiated ergosterol. In the case of the latter form, if there is any decrease in antirachitic effectiveness in the body of the chick it is no greater in the digestive tract than in the tissues. The body weights of the injected groups were considerably less than those of the fed groups.

2. Although the outward appearance of the group fed capsules of cod liver oil and that fed irradiated ergosterol was the same and in neither was there any external evidence of leg weakness, the bone ash percentage of the latter was significantly less than that of the cod liver oil-fed lot. Also the average body weight of the ergosterol group was significantly less at 7.5 weeks of age than that of the group which received cod liver oil.

3. The blood serum calcium values are of the order usually observed on an experimental régime of this type, but the marked difference in the bone ash percentage which occurred between the pairs of groups which received cod liver oil and irradiated ergosterol, respectively, did not prevail in the case of the calcium values. The inorganic phosphorus values for the blood serum are higher in the case of the capsule-fed groups than those of the corresponding injected groups and also higher than that usually obtained when an ample amount of the factor is mixed with the ration. Those for the injected and basal groups are considered normal. An explanation of this difference is not apparent at the present time.

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## THE EFFECT OF LIVER INJURY ON THE CONJUGATION OF BENZOIC ACID IN THE DOG\*

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In a recent study (1) it was found that the synthesis of hippuric acid is markedly diminished in various diseases of the liver especially those in which jaundice is present. These results are all the more interesting because in spite of the extensive research that has been done on hippuric acid, little attention has hitherto been given to the effect of liver damage. The discovery of Bunge and Schmiedeberg (2) that the synthesis of hippuric acid in the dog takes place only in the kidney, led to the tacit assumption that even in man the liver is of little importance in the detoxication of benzoic acid. Most workers, moreover, assumed that the formation of hippuric acid represented the sole conjugation that benzoic acid underwent in the organism; consequently, they ignored the combination with glycuronic acid, which in case of the dog led to a serious error.

Since the conjugation of benzoic acid in the human differs from that in the dog and other animals, it is not permissible to apply indiscriminately the data obtained on lower animals to man. A few studies on man have been made. In 1858, Kühne (3) reported that he could find no hippuric acid in the urine of a patient with obstructive jaundice. Zimmermann (4) found that after giving sidonal, a salt of quinic acid, to a patient with a biliary fistula, free benzoic acid but no hippuric acid appeared in the urine. Folwarczny (5) and more recently Snapper (6) found that hippuric acid is synthesized in patients with obstructive jaundice. Bryan (7) studied the synthesis of hippuric acid in a small series

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

of liver cases, and in over one-half of these patients he obtained a diminished rate of excretion. He seemed, however, much more impressed by the increase in the excretion of free benzoic acid, and apparently he underestimated the importance of his results.

The first animal experiments were reported by Kühne and Hallwachs (8) in 1857. They reported that hippuric acid was found in dogs with biliary fistulas, but that it was absent in the urine of cats after ligation of the common duct. These authors concluded that hippuric acid is formed in the vessels of the liver in the presence of bile constituents. Rosenberg (9) was able to find hippuric acid in the urine of a dog with a biliary fistula. Lewis (10) noted a diminished output in a rabbit with complete biliary drainage. Lackner, Levinson, and Morse (11) reported diminished excretion of hippuric acid after hydrazine poisoning, and Delprat and Whipple (12) stated that the synthesis of hippuric acid is greatly delayed after chloroform anesthesia. Both of the latter investigations were made on dogs, and hippuric acid was determined by the Folin-Flanders (13) method. The fact that this procedure determines total combined benzoic acid and not hippuric acid, invalidates their conclusions. Brakefield and Schmidt (14) studied the conjugation of benzoic acid in dogs after ligation of the common duct, and recorded the interesting finding that the production of benzoyl glycuronic acid was greatly reduced and the excretion of free benzoic acid proportionately augmented.

From the results recorded in Table I, it must be concluded that complete biliary obstruction has no demonstrable effect on the conjugation of benzoic acid. Contrary to the findings of Brakefield and Schmidt, no diminution in the production of glycuronic acid monobenzoate, nor any appreciable increase in the excretion of free benzoic acid was observed after ligation of the common duct. The present research differs from that of Brakefield and Schmidt only in that the sodium benzoate was incorporated in the food and fed, whereas these authors gave the drug parenterally. That the difference in results is not, however, due to the mode of administration is shown by the fact that in one dog (Dog 4) subcutaneous injection of sodium benzoate even on the 18th day of the obstruction gave normal results.

No explanation can be offered for the difference in the results obtained in the present study and those of Brakefield and

TABLE I  
*Effect of Obstructive Jaundice on Conjugation of Benzoic Acid*

Date	Dog No.	Weight	Benzoic acid excreted in 24 hrs.			Remarks		
			Free	Combined with				
				Glycuronic acid	Glycine			
1930		kg.	gm.	gm.	gm.			
Nov. 6	3	8				Ligation of common duct*		
" 8			0.05	3.68	1.42	5 gm. benzoic acid		
" 10			0.06	3.29	1.47	5 " " "		
" 13			0.08	2.66	1.34	5 " " "		
" 15			0.12	3.62	1.32	5 " " "		
" 17			0.07	3.40	1.48	5 " " " sub-cutaneously		
" 20	4	10				Ligation of common duct		
" 22			0.03	2.74	1.17	5 gm. benzoic acid; dog vomited		
" 24			0.03	3.83	1.60	5 gm. benzoic acid		
Dec. 8			0.04	2.33	0.85	3 " " " sub-cutaneously		
Nov. 17	W	12				Ligation of common duct		
Dec. 2			0.03	2.62	1.26	5 gm. benzoic acid		
Benzoic acid excreted combined with								
			Glycuronic acid			Glycine		
			6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.
			gm.	gm.	gm.	gm.	gm.	gm.
1931								
Jan. 9	5	7						
" 14	.		1.21			0.25		
" 16			1.22	1.35	0.95	0.19	0.40	0.32
" 20			0.51	0.36	0.47	0.42	0.29	0.80
" 26			1.67†	1.36	0.76	0.17	0.27	0.29
Ligation of common duct								
4 gm. benzoic acid								
5 " " "								
4 " phenylacetic acid								
5 " benzoic acid								

\* About 1 cm. of the common duct was resected. The success of the operation was verified by autopsy in all four dogs.

† 0.70 gm. of free benzoic acid in 24 hours. Urine contained blood and was strongly alkaline.

Schmidt. The fact that four dogs after ligation and resection of the common duct showed no diminution in the production of glycuronic acid monobenzoate, and only in one instance an appre-

cial excretion of free benzoic acid (which could be accounted for by the marked alkalinity of that particular specimen of urine) is fairly conclusive proof that obstructive jaundice *per se* does not greatly affect the conjugation of benzoic acid in the dog. It is doubtful whether it is possible to produce a condition in which the organism will excrete free benzoic acid. It should be again emphasized that both hippuric acid and glycuronic acid monobenzoate are readily hydrolyzed in alkaline solution; and in an alkaline urine enough hydrolysis can occur even in the bladder to give erroneous results.

It is fairly certain that glycuronic acid is closely related to carbohydrate metabolism and that its production is dependent upon an available supply of carbohydrate. Efforts were therefore made to maintain the animals in a good state of nutrition; forced feeding was resorted to whenever necessary. Since biliary obstruction of 2 to 3 weeks duration does not disturb the production of glycuronic acid, one is led to conclude that obstructive jaundice probably does not greatly affect carbohydrate metabolism. Whether obstruction of longer duration in which marked atrophy of the liver occurs will ultimately influence the synthesis of glycuronic acid, cannot be stated.

Light chloroform anesthesia greatly diminished the production of glycuronic acid. The output of glycuronic acid monobenzoate the day following a light 45 minute anesthesia was found to be definitely low (Table II). This is in accord with the findings of Delprat and Whipple, who also noted a delay in the excretion of conjugated benzoic acid, which they, however, erroneously called hippuric acid. As a matter of fact, the synthesis of hippuric acid is not appreciably influenced, which is to be expected, since in the dog this conjugation takes place only in the kidney. The effect of chloroform is only temporary, for in a few days the output of glycuronic acid again returned to normal. It should be mentioned that small doses of chloroform apparently stimulate the production of glycuronic acid, for on giving 1 to 2 cc. by stomach tube, the output of glycuronic acid monobenzoate was usually augmented. Further work is necessary, however, before any definite conclusions can be drawn. Whether the toxic action of chloroform, which sometimes terminates in acute yellow atrophy, is related to its depressing action on the synthesis of glycuronic acid cannot be answered, but offers an interesting speculation.

The effect of an Eck fistula on the conjugation of benzoic acid was found to be inconstant. In one dog, the glycuronic acid production dropped to about one-half 2 weeks after the operation

TABLE II

*Effect of Chloroform Anesthesia and of Eck Fistula on Conjugation of Benzoic Acid*

Date	Dog No.	Weight	Benzoic acid excreted combined with						Remarks
			Glycuronic acid			Glycine			
			6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.	
1950-51		kg.	gm.	gm.	gm.	gm.	gm.	gm.	
May 8	D	8	0.92	0.86	0.81	0.30	0.32	0.49	4 gm. benzoic acid
" 12			0.76	1.02	0.85	0.24	0.29	0.62	4 " " "
" 16									45 min. light chloroform anesthesia
" 17			0.50	0.55	0.88	0.19	0.27	0.70	4 gm. benzoic acid
" 18			0.86	0.72	0.82	0.29	0.18	0.47	4 " " "
Dec. 2	1	10							Eck fistula operation
" 9			1.17	1.15	0.75	0.27	0.27	0.76	5 gm. benzoic acid
" 15			0.30	0.27		0.43	0.50	1.29	5 " phenylacetic acid
" 18			0.53			0.28			5 " benzoic acid
" 22			0.61	0.61		0.30	0.34		5 " " "
" 26			0.58	0.77	0.91	0.32	0.34	0.68	5 " " "
Jan. 5			0.65	1.10	1.63	0.23	0.29	0.58	5 " " "
" 7									5 " phenylacetic acid
" 10			0.79	1.11	0.87	0.67	0.66	0.52	5 " benzoic acid and 15 gm. gelatin
" 26			1.13	1.01	0.90	0.34	0.38	0.60	5 gm. benzoic acid
Mar. 21	2	11							Eck fistula operation
" 31			1.55	1.27	0.94	0.33	0.32	0.55	5 gm. benzoic acid
Apr. 4			1.45	1.20	1.01	0.43	0.37	0.63	5 " " "
" 12			1.62						5 " " "
" 18			1.52	1.06	0.85				5 " " "

The results obtained on the third Eck fistula dog are not given in the table, since they were essentially the same as those obtained on Dog 2.

and remained at this low level for about 3 weeks, and then returned to normal. In two other dogs no such decrease in glycuronic acid was obtained. No change in the hippuric acid synthesis was noted in any of the Eck fistula dogs. From these results, it can be

concluded that the Eck fistula does not profoundly influence the conjugation of benzoic acid, and does not have any constant effect on the production of glycuronic acid.

#### SUMMARY

It was found that in dogs: (1) obstructive jaundice produced by ligation and resection of the common duct did not affect the conjugation of benzoic acid; (2) chloroform anesthesia caused a temporary decrease in the synthesis of glycuronic acid; (3) Eck fistula gave inconstant results, but apparently produced no permanent effect on the conjugation of benzoic acid.

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## THE RELATIONSHIP BETWEEN MUSCLE CREATINE AND CREATININE COEFFICIENT

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The proof that the creatinine output in the urine is related to the creatine concentration of muscle has been based on (1) the indirect method of comparing body weight or degree of musculature with the creatinine excretion and (2) by the more direct method of analyzing the muscle and urine in the same animal. The indirect procedure gives approximations which could not tolerate a statistical analysis. The method of direct analysis is based on a more substantial experimental basis but the results noted in the literature are variable, open to a number of interpretations, and can justifiably be criticized. In view of the uncertainty concerning the relationship of muscle creatine concentration to urinary creatinine, a series of well controlled experiments has been carried out on four species of animals.

The first reliable results recorded in the study of creatine-creatinine metabolism were made in 1904 when Folin (1) perfected a colorimetric method based on Jaffe's reaction. Folin studied the creatinine output in a number of normal individuals and noted that the daily creatinine excretion for a given individual was constant regardless of the amount of nitrogen excreted in the urine. Furthermore, it became obvious that of two individuals of approximately the same weight, the more obese person had a lower creatinine output per kilo of body weight. At this time he proposed the term creatinine coefficient to indicate the mg. of creatinine excreted daily per kilo of body weight. Shaffer (2), in 1908, noted that the daily creatinine output in a large number of individuals seemed to be roughly proportional to muscular development. In order to make comparative studies with other

nitrogenous constituents of the urine, Shaffer suggested that the creatinine coefficient be defined as the mg. of creatinine nitrogen excreted per kilo of body weight. McClugage, Booth, and Evans (3) found a relationship between muscular development and creatinine excretion in fourteen subjects. The patients that exhibited a high creatinine coefficient had well developed muscles, while those with a low coefficient had either poorly developed muscles or were excessively obese.

Garot (4) (quoted from Beard and Barnes (5)) stated that the creatinine excretion is not proportional to the muscular mass. In a preliminary statement, Beard (5) claimed to have evidence which showed a lack of any relationship between creatinine excretion and several body measurements in man.

The first attempt actually to study a direct relationship between muscle creatine and creatinine output was made by Myers and Fine (6). In this work the creatine content of cat, dog, human, and rabbit muscles was studied and was related to the creatinine coefficients in the dog, rabbit, and man. The experiments performed with rabbits were adequate in number. The creatine concentration of the muscle (abdominal and leg) of man was obtained from two autopsies and these analyses were compared with the creatinine coefficients of three normal men about 27 years of age. The creatinine coefficients of three dogs were compared with the muscle analyses obtained on five dogs.

Benedict and Osterberg (7) criticized the work of Myers and Fine on the ground that they took no account of the possible quantitative influence of adipose tissue. They suggested that in order to make a significant comparison, the creatinine coefficient should be determined when the organism is on a nitrogen-free carbohydrate diet; *i.e.*, when a minimum level of nitrogen excretion has been reached. These workers emphasized the necessity of taking into account such factors as ash, fat, and water in the analysis of tissue for creatine. They demonstrated that the "organic creatine" of the muscles of phlorhizinized dogs closely approximated similar results obtained in rabbits by Mendel and Rose (8). These data are extremely interesting in view of the erroneous conclusions that may be drawn as a result of wet tissue analyses. In 1914, Shaffer (9) contended that a study of the water

and nitrogen content of tissues might possibly account for the differences in the creatine content of muscles in different species.

Palladin (10) presented data for a number of species of animals which substantially confirmed the hypothesis put forward by Myers and Fine. These data however were partly drawn from sources other than his own experimental work. Terroine and Garot (11) studied the muscle creatine and the creatinine output of the rat, rabbit, dog, guinea pig, and horse. Using muscle analyses obtained in the literature from various sources, these workers concluded that urinary creatinine is not related to muscular mass. Conclusions drawn from data obtained from many sources cannot be taken too seriously.

It is obvious from this review of the literature that there is a difference of opinion regarding the possible relationship between creatinine output and muscle creatine. Furthermore, no effort has been made to test the statistical accuracy of such a relationship.

#### EXPERIMENTAL

The dogs, rabbits, rats, and guinea pigs used in these experiments were mature animals, carefully selected and in good condition. Care was taken that a balanced ration was always available. Each animal was allowed to become thoroughly accustomed to the diet fed and was not studied until a constant weight had been reached. The animals were placed in roomy metabolism cages and the urines were collected under toluene.

The dogs were placed on a creatine-free prepared dog food preparation (Bal Ra<sup>1</sup>). These animals were catheterized at a given time each day. Urine was collected for at least 3 days and for a longer period if the daily creatinine analyses did not check closely. At the termination of a satisfactory metabolism study, the dog was killed with an ether-chloroform mixture.

The rabbits were placed on a diet containing cracked corn, crushed oats, alfalfa meal, linseed meal, molasses, and 1 per cent salt. During the metabolism period, the rabbits were catheterized by gentle pressure over the bladder. Satisfactory creatinine analyses were usually obtained after 5 to 7 days and at the end

<sup>1</sup> Valentine Meat Juice Company, Inc., Richmond, Virginia.

of this time the animal was killed by a sharp blow on the back of the neck.

The rats used were mature stock animals obtained from our colony. They were fed a diet consisting of 20 per cent dried extracted beef, 15 per cent lard, 46 per cent corn-starch, 5 per cent cod liver oil, 5 per cent dried yeast, 4 per cent salt mixture (Osborne and Mendel (12)), 3 per cent sodium chloride, and 2 per cent agar. Daily urine collections were made for at least 7 days. Since no satisfactory method of catheterizing these animals can be used, it frequently became necessary to continue the metabolism experiment for as long as 2 weeks before constant results could be obtained. The metabolism cage used was similar to that of Levine and Smith (13). The rat was killed by a sharp blow on the neck.

The guinea pigs were fed fresh lettuce and were kept in metabolism cages for at least 6 days. The animal was killed by a blow on the neck.

In all these animals the thigh muscles were immediately dissected free of visible fat after death and the muscle was thoroughly ground and analyzed for creatine by the method of Rose, Helmer, and Chanutin (14). The creatinine in the urine of the rat was determined by the method of Folin and Morris (15), that of the other animals by the method of Folin (16). Total nitrogen determinations were made on approximately 2 gm. portions of tissue by the Kjeldahl method. The fat was estimated by extracting the dried tissue residue obtained in the total solid determination with ether in a Soxhlet apparatus. The muscle was incinerated in a muffle furnace for ash. All of these analyses were carried out in duplicate for the rat and the guinea pig and in triplicate for the rabbit and the dog.

Our data were treated by accepted statistical methods and the standard deviations and the correlation coefficients estimated. The formula for standard deviation was

$$\sqrt{\frac{\sum d^2}{N} - C^2 \cdot \text{step interval}}$$

in which  $\sigma$  = the deviation from the average,  $d^2$  = the sum of the squared deviations in units of step intervals,  $C^2$  = the correction in units of step intervals,  $N$  = number of animals.

For the correlation coefficient and its probable error the following formulæ were used.

$$r = \frac{\sum xy}{\sqrt{\sum x^2} \cdot \sqrt{\sum y^2}}, \text{ probable error } r = 0.675 \cdot \frac{1 - r^2}{n}$$

in which  $r$  = the correlation coefficient,  $x$  = the deviation of any item of the first series from the average of the series,  $y$  = the deviation of any item of the second series from the average of the series,  $x^2$  and  $y^2$  = the sums of the squared deviations from the two averages,  $n$  = the number of items.

Organic creatine refers to the creatine in the dried, ash- and fat-free muscle.

### Results

*Muscle Creatine and Creatinine Coefficient of Dog, Guinea Pig, Rabbit, and Rat*—The results obtained with seven dogs are summarized in Table I. It is seen that the wet and organic creatine concentrations are variable. These results approximate those obtained by Folin and Buckman (17) and Janney and Blatherwick (18) but differ markedly from the many analyses tabulated by Hunter (19). The creatinine coefficients for the dog as listed in Table I are uniformly higher than those listed in the literature (19). There is no comparative uniformity of data when one studies the creatine of the muscle and the creatinine coefficients in the individual animals. The correlation coefficients for these two factors are  $-0.210 \pm 0.244$  and  $-0.461 \pm 0.200$  for the wet and organic creatine, respectively.

The analytical data for twenty-one guinea pigs are also presented in Table I. The greatest individual variations in muscle creatine were obtained in this species. The creatinine coefficients were also variable and showed no relationship to either the wet or organic creatine determinations in individual cases. The figures obtained for creatine and the creatinine coefficient are higher than those listed by Hunter (19). A statistical study shows a correlation coefficient of  $-0.101 \pm 0.146$  (wet) and  $0.036 \pm 0.147$  (organic) between muscle creatine and the creatinine coefficient. It should be pointed out that there is a marked difference in the amount of ether-soluble material in the muscles

TABLE I

*Muscle Creatine and Creatinine Coefficient of Dog and Guinea Pig*

In these experiments female dogs and male guinea pigs were used.

	Dead weight	Ash	Total solids	Fat	Creatine		Total N		Creatinine coefficient
					Wet	Organic	Wet	Dry	
	gm	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Dog 1	16,400	1 05	25 1	2 10	0 457	2 08	3 38	15 4	14 4
" 2	19,650	1 03	25 2	1 88	0 412	1 85	3 46	15 5	16 0
" 3	9,750	1.09	24 8	3 11	0 406	1 97	3 23	15 7	12 0
" 4	10,830	1 15	24 5	1 98	0 444	2 08	3 41	16 0	11 2
" 5	7,710	1 16	25 7	3 12	0 452	2 11	3 36	15 7	12 1
" 6	18,000	1 10	24 8	2 39	0 442	2 07	3 35	15 7	12 7
" 7	9,970	1 11	23 1	2 03	0 451	2 25	3 16	15 8	13 2
Mean		1 10	24 7	2 37	0 438	2 06	3 34	15 7	13 1
Standard deviation					±0 02	±0 11			±1 5
68 per cent of all cases will fall within					0 418- 0 458	1 95- 2 17			11 58- 14 62
Guinea Pig 1	618	1 25	23 8	1 39	0 565	2 66	3 30	15 7	18 8
" " 2	493	1 39	24 5	1 22	0 582	2 66	3 42	15 6	23 4
" " 3	483	1 08	24 4	1 69	0 568	2 63	3 40	15 7	22 4
" " 4	474	1 30	23 9	1 45	0 583	2 76	3 38	15 8	19 8
" " 5	417	1 34	24 1	1 06	0 528	2 44	3 46	16 0	21 7
" " 6	450	1 32	23 9	0 80	0 585	2 68	3 34	15 3	20 7
" " 7	322	0 74	23 4	0 10	0 592	2 62	3 46	15 4	18 2
" " 8	470	1 21	23 2	0 89	0 613	2 90	3 20	15 2	16 8
" " 9	370	1 25	23 0	0 86	0 565	2 70	3 22	15 4	17 8
" " 10	428	1 35	23 7	0 32	0 597	2 71	3 44	15 7	19 6
" " 11	546	1 22	23 9	1 25	0 598	2 78	3 29	15 3	15 4
" " 12	487	1 36	22 9	0 28	0 648	3 20	3 21	15 1	21 6
" " 13	524	1 30	23 5	1 08	0 604	2 86	3 33	15 7	19 5
" " 14	515	1 32	22 6	0 34	0 630	3 01	3 35	16 0	22 1
" " 15	516	1 30	22 8	0 44	0 576	2 73	3 27	15 5	18 3
" " 16	570	1 36	22 5	0 95	0 605	3 00	3 18	15 8	18 0
" " 17	527	1 26	23 4	0 88	0 580	2 73	3 30	15 5	19 4
" " 18	604	1 29	23 3	1 32	0 590	2 85	3 24	15 7	23 4
" " 19	543	1 26	22 4	1 11	0 576	2 88	3 15	15 7	24 4
" " 20	587	1 36	23 8	1 31	0 615	2 91	3 33	15 8	20 2
" " 21	706	1 26	23 6	1 36	0 580	2 76	3 28	15 6	21 2
Mean		1 26	23 5	0 95	0 585	2 77	3 31	15 6	20 1
Standard deviation					±0 025	±0 17			±2 36
68 per cent of all cases will fall within					0 560- 0 610	2 6- 2 9			17 7- 22 5

TABLE II  
Muscle Creatine and Creatinine Coefficient of Rabbit and Rat

		Dead weight	Ash	Total solids	Fat	Creatine		Total N		Creatinine coefficient
						Wet	Organic	Wet	Dry	
		gm	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Rabbit	1 ♂	2890	1 24	24 6	0 615	0 523	2 30	3 71	16 3	19 7
"	2 ♂	2890	1 22	24 3	0 756	0 499	2 24	3 60	16 2	17 5
"	3 ♂	3240	1 22	24 6	0 812	0 521	2 31	3 65	16 2	19 0
"	4 ♂	3060	1 24	24 0	0 718	0 526	2 38	3 53	16 0	20 3
"	5 ♂	3160	1 23	24 3	0 539	0 526	2 34	3 64	16 1	19 3
"	6 ♂	3040	1 23	24 2	0 881	0 533	2 41	3 62	16 4	16 0
"	7 ♂	2570	1 24	24 9	0 794	0 501	2 18	3 71	16 2	22 6
"	8 ♂	3330	1 26	25 3	1 02	0 489	2 13	3 67	16 0	17 3
"	9 ♂	2760	1 27	24 6	0 820	0 484	2 15	3 57	15 9	20 2
"	10 ♂	2600	1 17	24 7	0 740	0 503	2 20	3 60	15 8	19 4
"	11 ♂	2890	1 24	24 3	0 524	0 551	2 44	3 58	15 9	18 1
Mean			1 23	24 5	0 744	0 514	2 28	3 63	16 1	19 1
Standard deviation						±0 02	±0 10			±1 66
68 per cent of all cases will fall within						0 494-	2 18-			17 54-
						0 534	2 38			20 66
Rat	1 ♂	320	1 17	24 8	2 05	0 446	2 06			15 5
"	2 ♀	234	1 26	26 2	3 27	0 457	2 12			14 9
"	3 ♂	400	1 27	24 7	3 24	0 461	2 30			15 4
"	4 ♀	206	1 19	26 4	2 25	0 500	2 17			17 4
"	5 ♀	244	1 22	26 3	2 50	0 491	2 17			18 5
"	6 ♂	346	0 88	25 2	1 83	0 478	2 12	3 44	15 3	14 9
"	7 ♂	406	1 28	26 5	3 88	0 485	2 28	3 39	15 9	12 6
"	8 ♀	256	1 12	25 7	2 81	0 483	2 22	3 43	15 7	13 1
"	9 ♂	320	1 26	26 4	3 45	0 505	2 32	3 49	16 1	14 4
"	10 ♂	312	1 10	25 1	2 03	0 506	2 31	3 53	16 1	15 2
"	11 ♀	217	1 21	25 8	1 98	0 508	2 25	3 58	15 8	16 7
"	12 ♀	242	1 17	25 4	1 81	0 509	2 27	3 59	16 0	13 1
"	13 ♂	348	1 90	24 8	1 97	0 464	2 22	3 46	16 5	12 9
"	14 ♀	256	0 63	24 9	1 65	0 487	2 15	3 53	15 6	14 3
"	15 ♂	325	1 14	25 7	2 96	0 453	2 10	3 41	15 8	12 3
"	16 ♂	289	0 42	26 2	3 27	0 461	2 05	3 47	15 4	12 5
"	17 ♂	337	0 60	24 2	1 67	0 444	2 02	3 50	16 0	12 0
Mean			1 10	25 5	2 51	0 479	2 18	3 49	15 9	14 4
Standard deviation						±0 022	±0 10			±1 8
68 per cent of all cases will fall within						0 457-	2 08-			12 6-
						0 501	2 28			16 2

of these animals which does not necessarily have any relationship to the weight of the animal at death.

The data for eleven rabbits shown in Table II show the individual differences for both the muscle creatine and the relationship between creatine and creatinine coefficients. The correlation coefficients are  $-0.241 \pm 0.192$  (wet) and  $-0.317 \pm 0.183$  (organic). The mean value obtained for the mixed white thigh muscle of the rabbit is almost identical with the value obtained by Myers and Fine (6) and others (19). The creatinine coefficients obtained are slightly higher than many of the figures listed by Hunter.

In Table II are also given the data for seventeen rats which are of the same general type noted for the dog, guinea pig, and rabbit. The correlation coefficients are  $0.409 \pm 0.136$  (wet) and  $0.177 \pm 0.159$  (organic) for the muscle creatine and the creatinine coefficients. These data for muscle creatine and creatinine coefficient in individual animals approximate the results obtained by many other investigators.

#### DISCUSSION

A study of the literature dealing with the creatine concentrations in muscles of various species shows tremendous variations in the results obtained. Several workers, particularly Myers and Fine (6), have been able to obtain unusually constant analyses for the rabbit, dog, and rat (20) using the extraction method for the determination of creatine. On the other hand, Folin and Buckman (17) obtained extremely variable results with muscle creatine in individual species with the use of a method that involved the direct extraction and conversion of creatine to creatinine in the autoclave. These workers concluded "that the creatine contents of the muscles of cats, rabbits, and hens vary within substantially the same limits. The variations found appear to be too large to permit the use of average figures in calculations as to the alleged relationship between the creatinine elimination and the total amount of creatine in the tissues." In the results given in this paper, we cannot confirm the great variations found for rabbit and dog muscles nor could we obtain the degree of constancy noted by Myers and Fine. Our results obtained with the guinea pig are extremely variable and showed the largest range

between the minimum and maximum results. Our data show that the standard deviations obtained for the dog, rat, rabbit, and guinea pig were almost identical, thus indicating a very constant variation in creatine concentration.

Myers and Fine (6) have concluded that the "creatinine elimination appears to bear a distinct relation to the percentage content of muscle creatine in a given species." They further stated that, "The constancy in the content of muscle creatine offers a satisfactory explanation for the constancy in the daily elimination of creatinine." The mean values obtained by these workers for twenty rabbits, two men, and five dogs seem to bear out the conclusion of a relationship between creatinine output and muscle creatine concentration. However, a statistical analysis of their data for the creatine concentration in fresh muscle and creatinine coefficient in rabbits gives a correlation coefficient of  $0.263 \pm 0.190$ . If this method of analysis can be depended upon, the interpretation and conclusions of Myers and Fine cannot be correct.

Inspection of the mean values obtained in these experiments for the creatine concentrations of muscle and creatinine coefficients for different species would lead one to believe that the conclusions of Myers and Fine were correct. The standard deviation from the averages for these animals is almost identical in the case of the wet creatine. However, when one studies the statistical reliability between the creatine concentration of muscle and the creatinine coefficient in individual animals, one is impressed by the total lack of any relationship between these factors, particularly when the correlation coefficients for both wet and "organic" creatine are considered. Statistical analysis of "organic" creatine of guinea pig muscle reveals a larger variation in standard deviation than obtained in the dog, rat, and rabbit. The reason for this difference can be attributed to the great variations in the fat content of guinea pig muscle. Furthermore, the standard deviations obtained for creatinine coefficients are relatively large and variable in the animals studied and must be responsible to a great extent for the poor correlations obtained. Statistical methods do not permit a comparison of averages for any interpretation unless the variations are within reliable ranges.

## SUMMARY

These results demonstrate that there is a true difference in the creatine concentrations of the wet muscle of the dog, rat, rabbit, and guinea pig. The organic creatine concentrations for the dog, rabbit, and rat are almost identical but the results for the guinea pig are distinctly higher.

A statistical analysis of the data obtained in the dog, rat, rabbit, and guinea pig indicates an absence of any relationship between creatinine elimination and the percentage concentration of muscle creatine.

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# THE FORMATION OF A HOMOLOGUE OF CYSTINE BY THE DECOMPOSITION OF METHIONINE WITH SULFURIC ACID\*

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(Received for publication, October 15, 1932)

Methionine upon being heated with strong sulfuric acid was found to give a positive reaction with the Folin-Marenzi (1) reagent for cystine. This observation was made in connection with another investigation which had necessitated a study of the behavior of the individual amino acids towards this reagent, after they had been heated with acid. Under the conditions of ordinary protein analysis only a slight reaction was obtained with methionine but as the concentration of sulfuric acid was increased to about 12 N a strong positive test resulted. At a concentration of 18 N the maximum reaction was obtained, giving as much color as would be given by four-tenths as much cystine.

It was first thought that dimethyl disulfide was the cause of this reaction as its odor was very noticeable, but it soon became apparent that only relatively small amounts of this compound were present and that the material responsible for the reaction was non-volatile. The conditions for this interesting decomposition were, therefore, studied in detail and the isolation and identification of the non-volatile cystine-reacting substance undertaken.

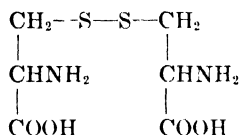
A sulfur-containing nitrogenous compound was finally isolated from the reaction mixture, which accounted for the major portion of the material which had given the positive Folin-Marenzi reaction. It crystallized from water in irregular thin hexagonal plates and decomposed without melting at 260–265°. The sulfur was

\* A preliminary report of this work was presented before the meeting of the American Society of Biological Chemists at Philadelphia, April 27–30, 1932.

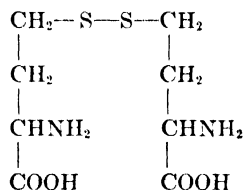
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shown to be present in the disulfide form since positive nitroprusside and Folin-Marenzi tests were obtained only after reduction. The sulfur could also be split off as lead sulfide when the compound was heated with sodium hydroxide and lead acetate. From the elementary analysis for carbon, hydrogen, sulfur, and nitrogen the minimum empirical formula on the basis of the presence of a disulfide grouping was calculated to be  $C_8H_{16}O_4N_2S_2$ . By means of the Van Slyke method it was demonstrated that all the nitrogen was present as amino nitrogen. Since the compound was amphoteric, dissolving in both acids and bases, and since the ninhydrin reaction was positive, it was decided that the compound must be a disulfide amino acid.

From the above facts and from a consideration of the possible decomposition products of methionine we have drawn the conclusion that the compound is bis-( $\gamma$ -amino- $\gamma$ -carboxypropyl) disulfide, the next higher symmetrical homologue of cystine. Because of this relationship we wish to suggest the name of homocystine for the compound. This relationship is shown by the following formulas.



Cystine



Homocystine

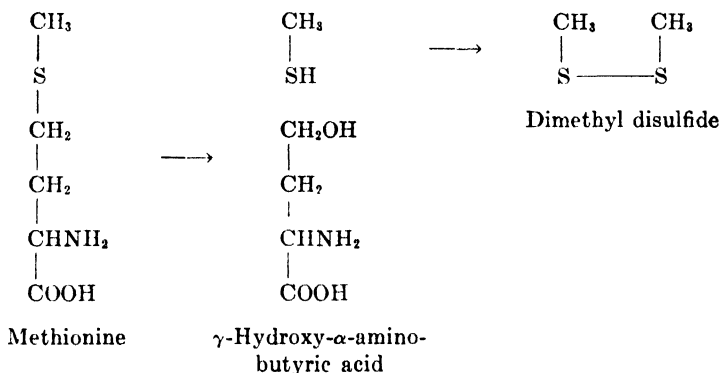
This conclusion was confirmed by the preparation of a number of different types of derivatives. By benzoylation with benzoyl chloride by the Schotten-Baumann reaction we obtained a dibenzoyl derivative in accordance with what would be expected from the above formula. By reduction in liquid ammonia with metallic sodium and treatment with benzyl chloride according to the method of du Vigneaud, Audrieth, and Loring (2) a benzyl derivative was obtained, the analysis of which agreed with  $\gamma$ -benzylthio- $\alpha$ -aminobutyric acid,  $C_6H_5CH_2-S-CH_2-CH_2-CHNH_2-COOH$ . By oxidation with bromine water a sulfonic acid was isolated agreeing in composition and properties with what would be expected of the next higher homologue of cysteic acid.

The fact that all these derivatives agreed in composition with the theoretical values expected convinced us that the structure given above is the correct one for this compound. It is realized, however, that an absolutely final decision must await synthetic confirmation which we hope to accomplish.

Since the homocystine was prepared from *dl*-methionine, it is apparent from the structural formula given above that the compound isolated could be either the racemic or the meso modification of inactive homocystine. We have as yet no evidence which stereoisomer the compound isolated represents.

Its behavior in the Folin-Marenzi method is in agreement with the structure assigned to the compound. In comparison with cystine the degree of color development is inversely proportional to the molecular weights. The response of homocystine to the Sullivan test (3) for cystine was very interesting in that although homocystine is so closely related in structure to cystine, a negative reaction was obtained.

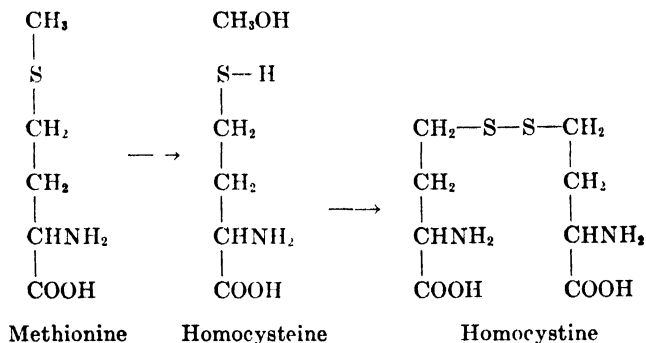
In the decomposition of methionine the presence of a small amount of dimethyl disulfide can be explained on the basis of a hydrolysis of the methionine yielding  $\gamma$ -hydroxy- $\alpha$ -aminobutyric acid and methyl mercaptan. The latter compound could then be oxidized to the disulfide. This decomposition can be represented by the following equations.



If the hydrolysis, however, should take place between the methyl group and the sulfur then methyl alcohol and homocysteine

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would result and the latter on oxidation would yield the disulfide homocystine in the following manner.



It is also conceivable that if both methyl mercaptan and the  $\gamma$ -thio- $\alpha$ -aminobutyric acid are present simultaneously, oxidation could also produce the mixed disulfide,  $\text{CH}_3-\text{S}-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ .

We have so far been unable to detect any free methyl alcohol either in the reaction mixture or in the distillate. It is indeed doubtful whether methyl alcohol if formed would remain unchanged under the conditions of the decomposition, secondary reactions probably taking place.

We have also studied the behavior of methionine with other acids. No such decomposition was found with  $\text{HCl}$ , but  $\text{HBr}$  and  $\text{HI}$  were found to bring about the decomposition, but to a lesser degree than did  $\text{H}_2\text{SO}_4$ .

The possible significance of this decomposition of methionine with respect to the determination of cystine in protein analysis cannot as yet be evaluated. Although the strength of acid necessary to produce the maximum amount of disulfide is far greater than that ordinarily used in protein analysis, yet small amounts of the substance are produced with weaker acid concentrations. Furthermore, it is conceivable that the stability of the sulfur of methionine which is linked with other amino acids in the protein molecule may be different from that of methionine in the free state. The stability of the sulfur of cystine for example is considerably influenced by the presence of other groups attached to the amino group (4).

A study of the growth-promoting properties of homocystine on a cystine-deficient diet should yield suggestive data on the possible intermediary steps of the metabolism of methionine in the body. Jackson and Block (5) have already shown that methionine will support growth on a cystine-deficient diet and if the utilization proceeds through a preliminary demethylation then homocystine should also be expected to support growth under similar conditions.

That homocystine itself might be present in proteins is a possibility that should be borne in mind and will be worth investigating.

#### EXPERIMENTAL

*Preparation of Homocystine*—17 gm. of *dl*-methionine were dissolved in 140 cc. of 18 N  $\text{H}_2\text{SO}_4$ . The solution was heated in a flask equipped with a water-cooled condenser for 8 hours in an oil bath kept at 125–135°. During the heating, nitrogen which had been passed through a heated tube containing copper was allowed to bubble through the solution. At the end of this heating period the solution was cooled and poured into 3 liters of water. Powdered  $\text{Ba}(\text{OH})_2$  was then added slowly with stirring. After the bulk of  $\text{BaSO}_4$  had been removed, the solution was quantitatively freed from both barium and sulfate ions. The combined  $\text{BaSO}_4$  precipitates were very thoroughly extracted with boiling water until the wash water no longer gave a Folin-Marenzi test. The combined filtrates were then concentrated *in vacuo* until the homocystine began to separate. The solution was then cooled in an ice bath to allow the homocystine to crystallize out. After the filtration of this crop of crystals the mother liquor was further reduced in volume to about 30 cc. and another crop of homocystine was obtained. The filtrate was alkaline to phenolphthalein showing that an even more deep seated decomposition of the methionine had taken place. The mother liquor still gave a very strong Folin-Marenzi reaction. As already indicated, two inactive forms of homocystine should be expected and it is possible that the other stereoisomer, which might be much more soluble, could be present in this mother liquor.

The combined crops of crystals were then recrystallized from water, a yield of 4.9 gm. being obtained which was 28.8 per cent of the theoretical amount. Colorimetric analysis by the Folin-Marenzi method of the original solution after heating indicated

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an amount of disulfide present which calculated as homocystine would have been 42.5 per cent of the theoretical amount possible. Variation of acid concentration and time of heating gave no higher yields.

The homocystine crystallized in thin irregular hexagonal plates as shown in Fig. 1. The solubility in water at 25° was found to be about 1 part in 5000. In an open capillary tube it decomposed without melting at 260–265°. The point of maximum insolubility of homocystine was found to be between pH 5.5 and 7.0, the compound dissolving in either an excess of acid or base. The substance gave a positive ninhydrin reaction and a positive nitroprusside test after reduction with NaCN. By means of the Van



FIG. 1. Homocystine crystals.  $\times 215$

Slyke amino nitrogen method, 10.05 per cent of amino nitrogen was obtained compared to the theoretical amount of 10.45.

### *Analysis*

4.569 mg. substance: 6.02 mg.  $\text{CO}_2$  and 2.55 mg.  $\text{H}_2\text{O}$

3.103 " " : 0.294 cc. N at 29.5° and 742 mm.

4.600 " " : 8.02 mg.  $\text{BaSO}_4$

$\text{C}_8\text{H}_{16}\text{O}_4\text{N}_2\text{S}_2$ . Calculated. C 35.82, H 5.97, N 10.45, S 23.88

Found. " 35.94, " 6.24, " 10.43, " 23.95

*Preparation of Dibenzoylhomocystine*—To 0.5 gm. of homocystine dissolved in 5 cc. of water and 7.15 cc. of 2.6 N  $\text{NaOH}$ , 1.07 cc. of benzoyl chloride were slowly added. After the mixture was shaken vigorously for a short time it was allowed to stand in the

refrigerator overnight. It was then acidified to Congo red with  $\text{H}_2\text{SO}_4$ . The crystalline precipitate which separated was filtered and extracted with 10 cc. portions of boiling benzene until all the benzoic acid had been removed. The crude dibenzoylhomocystine which weighed 0.9 gm. was recrystallized from absolute ethyl alcohol. The product melted at  $184\text{--}185^\circ$  (corrected).

*Analysis*

4.278 mg. substance:	8.73 mg. $\text{CO}_2$ and 1.92 mg. $\text{H}_2\text{O}$
3.138 " "	: 0.167 cc. N at $28.5^\circ$ and 745 mm.
4.392 " "	: 4.27 mg. $\text{BaSO}_4$
$\text{C}_{22}\text{H}_{24}\text{O}_6\text{N}_2\text{S}_2$ .	Calculated. C 55.40, H 5.08, N 5.88, S 13.44
	Found. " 55.66, " 5.02, " 5.90, " 13.35

*Preparation of dl-S-Benzylhomocysteine*—0.5 gm. of homocystine was slowly added to a slight excess of metallic sodium dissolved in dry liquid ammonia. Solid  $\text{NH}_4\text{Cl}$  was then added very gradually until the blue color due to the excess sodium was discharged. The ammonia was then allowed to evaporate spontaneously and the residue dissolved in a minimum quantity of water. The solution gave an intense nitroprusside test. To this solution was added 0.63 gm. of benzyl chloride and the mixture was shaken for 1 hour. At the end of this time the nitroprusside test was negative. The solution was then extracted with successive portions of ether and acidified with acetic acid. Colorless glistening crystals separated which were filtered and dried, a yield of 84 per cent being obtained. Recrystallization from water gave irregular plates melting at  $190\text{--}191^\circ$  (corrected).

*Analysis*

4.376 mg. substance	9.33 mg. $\text{CO}_2$ and 2.63 mg. $\text{H}_2\text{O}$
3.068 " "	0.169 cc. N at $27.0^\circ$ and 747 mm.
4.198 " "	4.33 mg. $\text{BaSO}_4$
$\text{C}_{11}\text{H}_{15}\text{O}_2\text{NS}$ .	Calculated. C 58.70, H 6.71, N 6.23, S 14.20
	Found. " 58.15, " 6.72, " 6.16, " 14.16

*Preparation of dl-Homocysteic Acid*—To 0.5 gm. of homocystine suspended in 10 cc. of water liquid bromine was added cautiously until the yellow color due to the bromine disappeared only sluggishly. Bromine water was then added until a faint yellow color remained for about an hour. The resulting solution was concentrated at room temperature in a current of air and the residue placed in a vacuum desiccator containing anhydrous and sodium

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hydroxide for several days. To the thick syrup that was so obtained absolute ethyl alcohol was added. A colorless crystalline product was precipitated, which after repeated extractions with absolute ethyl alcohol was recrystallized from 25 per cent ethyl alcohol. The resulting homocysteic acid decomposed at 230–235° and was obtained in the form of small poorly defined crystals. The yield was 42 per cent of the theoretical.

### *Analysis*

4.790 mg. substance: 4.65 mg. CO<sub>2</sub> and 2.20 mg. H<sub>2</sub>O

3.182 “ “ : 0.225 cc. N at 32.5° and 748 mm.

4.494 “ “ : 5.720 mg. BaSO<sub>4</sub>

C<sub>4</sub>H<sub>9</sub>O<sub>3</sub>NS. Calculated. C 26.22, H 4.92, N 7.69, S 17.48

Found. “ 26.47, “ 5.14, “ 7.76, “ 17.48

The authors wish to thank Mr. Kurt Eder, micro analyst of this laboratory, for carrying out the micro analyses.

### SUMMARY

Methionine upon being heated with 12 to 18 N sulfuric acid gives rise to a disulfide amino acid which has been isolated in crystalline form and identified as bis-(γ-amino-γ-carboxypropyl) disulfide, the next higher symmetrical homologue of cystine. The name homocystine has been suggested because of its relationship to cystine.

Dibenzoylhomocystine, benzylhomocysteine, and homocysteic acid have also been prepared.

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# THE TEMPORARY FORMATION OF THE AZLACTONE RING IN THE RACEMIZATION OF ACYL DERIVATIVES OF AMINO ACIDS WITH ACETIC ANHYDRIDE

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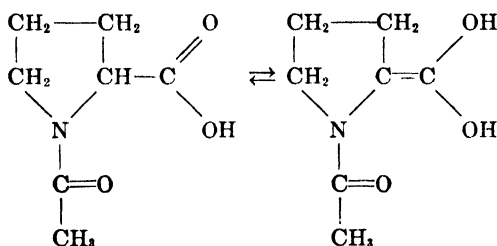
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It has been shown in a previous investigation (1) that the racemization of the sodium salt of acetyl-*l*-tryptophane in aqueous solution by acetic anhydride encountered by du Vigneaud and Sealock (2) is a reaction which can be extended to other amino acids. The reaction seems, in fact, to be a general one for primary  $\alpha$ -amino acids and can be used for the preparation of the racemic modification of these amino acids. In the case of acetylcystine, however, some decomposition accompanied the racemization of the compound, free sulfur and hydrogen sulfide being formed. Proline which contains a secondary amino group was not racemized by this treatment. It was also shown that the formyl as well as acetyl derivatives of amino acids could be racemized by this method.

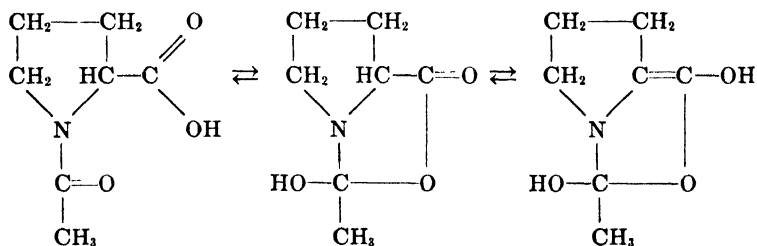
The mechanism of this reaction attracted our interest because of the mildness of the conditions under which the reaction takes place and the fact that it occurs in aqueous solution. It seemed reasonable to assume that the racemization was brought about either by some catalytic effect or by some definite reaction involving the groupings attached to the asymmetric carbon atom in contrast to the type of racemization such as that caused by boiling acid. Such reactions as the latter are generally regarded as occurring without a change in constitution of the molecule other than that of the arrangement of the groups around the asymmetric atom, this change being due merely to the increased vibration of these groups. In the racemization reaction we have under con-

sideration some chemical reaction undoubtedly occurs with the formation of an intermediary molecule in which the asymmetry is destroyed.

A keto-enol shift might seem at first sight to explain our racemization reaction, but the fact that we were unable to obtain racemization with acetylproline seems to us to be a strong argument against such an assumption. There is no convincing reason why acetylproline should not give a keto-enol shift as indicated in the following equation as well as any other amino acid since it has a hydrogen atom on the  $\alpha$ -carbon atom.

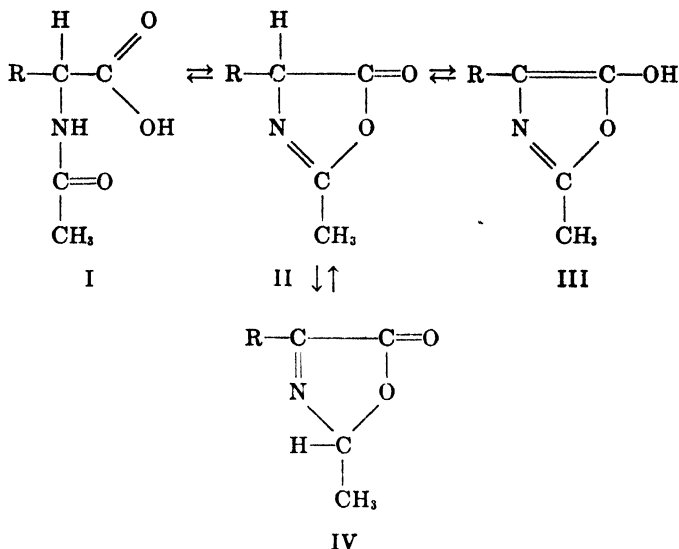


The failure of this compound to racemize would also eliminate as an explanation of our reaction the following keto-enol rearrangement suggested by Bergmann and Zervas (3) for their racemization reaction, since acetylproline would be expected to behave similarly to other amino acids in this respect.



A possible mechanism which seems to us to agree with the experimental evidence is one that is based upon a transitory formation of the azlactone of the acylated amino acid. It is known that whenever an azlactone of an amino acid which contains a hydrogen atom on the  $\alpha$ -carbon atom is formed, racemization results. This may be due to the migration of the hydrogen atom as represented

by the shift from (II) to (III) or to the carbon of the acyl grouping as indicated by the conversion of (II) to (IV).



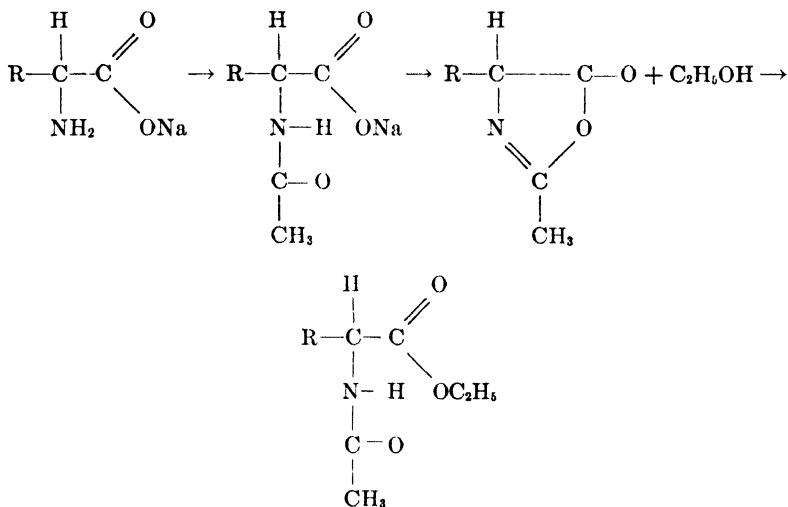
Either hydrogen atom shift in the azlactone ring would explain our racemization. Further the necessity of the formation of the azlactone in our racemization would explain the lack of racemization of proline since a true azlactone of proline or of any amino acid with a secondary amino group cannot be formed.

Since the behavior of proline is quite crucial for this theory, we felt that the behavior of some substituted amino acids besides proline should be investigated. We therefore synthesized N-methyl-*l*-phenylalanine and subjected it to our racemization reaction. Even though the reaction was allowed to continue for 96 hours, the optical activity was unaffected. N-Methyl-*l*-phenylalanine, like proline, should not be capable of yielding an azlactone.

The fact that racemization of acetyl-*l*-cystine is attended by decomposition and splitting out of sulfur seems to us to fit in well with this particular mechanism. It might be expected from the work of Bergmann and Delis (4) and Bergmann, Andrews, and Andrews (5) on the stability of various ring derivatives of cystine and serine that the azlactone of cystine would be unstable. The

transitory formation of the azlactone in our racemization reaction could therefore give rise to such decomposition.

The explanation we have offered for this mechanism has a close analogy in the theory postulated by Ashley and Harington (6) for their simultaneous acetylation-esterification reaction of amino acids. In this reaction the ethyl ester of the acetylated amino acid was obtained by treating the sodium salt of the amino acid in ethyl alcohol with acetic anhydride. Ashley and Harington explained this on the basis of a temporary formation of an azlactone which reacted with the ethyl alcohol to yield an ester according to the following equations.



In an analogous manner in our reaction the acetyl derivative might be dehydrated to give temporarily an azlactone which in turn would react with another molecule of water to give back again the acetylamino acid which would then be optically inactive.

It is conceivable that this esterification might occur simply through the dehydrating action of acetic anhydride by the splitting out of a molecule of water between the alcohol and the carboxyl group. Such an experiment was tried with hydrocinnamic acid in place of phenylalanine in Harington's reaction. A 30 per cent yield of ethyl hydrocinnamate was obtained. An experiment was also run in which glacial acetic acid was substituted for the acetic

anhydride. Although the attempted isolation of the ester was carried out by exactly the same technique as above, no ester was obtained, showing that the ester was not formed through any other step in the procedure and that the esterification was really due to the action of the acetic anhydride in this instance.

It is quite apparent that if the azlactone is temporarily formed in Harington's reaction with phenylalanine and thyroxine, racemization should result even as it did in our racemization reaction. This point is indeed a crucial one for their azlactone theory. Since the analogy to our reaction is so close and since we wished to utilize it as further evidence in favor of the temporary formation of the azlactone in our racemization, we decided to investigate more closely the esterification reaction. Since Ashley and Harington started with inactive phenylalanine, the acetyl ester they obtained was of course the inactive one and no decision could be drawn from their work as to whether or not racemization could have occurred. The thyroxine employed by them in the preparation of the acetyl ethyl ester of thyroxine was also probably the racemic modification. To reach a decision on this question we resolved synthetic phenylalanine and subjected the active amino acid to their procedure. The inactive acetyl ester of phenylalanine was obtained, demonstrating that racemization had occurred in the reaction. Further proof was therefore afforded in favor of the azlactone theory that Ashley and Harington had offered in explanation of their reaction.

If the azlactone formation should represent the true mechanism of this reaction, then proline should be incapable of yielding the racemized ester by this treatment. When *l*-proline was tried, only acetyl-*l*-proline could be isolated and we could obtain no evidence of either esterification or racemization.

#### EXPERIMENTAL

*Ethyl Ester of Acetyl-dl-Phenylalanine from d-Phenylalanine*—1.65 gm. of *d*-phenylalanine having a specific rotation of  $33.46^\circ$  were dissolved in 30 cc. of absolute alcohol containing 0.23 gm. of sodium. 5 cc. of acetic anhydride were added and the mixture shaken. The flask was attached to a reflux condenser, for the heat of the reaction is sufficient to cause the alcohol to boil. After 30 minutes, the alcohol and as much as possible of the acetic anhy-

dride were removed by vacuum distillation. The residue was extracted with about 40 cc. of ether and this solution washed in a separatory funnel with dilute  $\text{Na}_2\text{CO}_3$  solution to remove acetic anhydride still present. The ether layer was evaporated to dryness and 0.7 gm. of material was obtained. It was recrystallized from a mixture of equal parts of ethyl ether and petroleum ether (b.p. 65–110°). The melting point was 66–67° which agrees with the melting point of 67° reported by Ashley and Harington (6). The compound possessed no rotation whatsoever. The ester was likewise prepared starting with inactive phenylalanine and the melting point of the mixed products was taken. No lowering in melting point was detected.

*Attempted Formation of Ethyl Ester of Acetylproline*—5 gm. of *l*-proline were dissolved in 100 cc. of absolute alcohol containing 1.0 gm. of sodium. 20 cc. of acetic anhydride were added. The reaction mixture became slightly warm. It was allowed to stand 2 hours and was then reduced by vacuum distillation to a viscous syrup. This syrup was extracted with chloroform, in which the ethyl ester of acetylproline is readily soluble (7). Evaporation of the chloroform left no residue.

7.24 cc. of 6 *N*  $\text{H}_2\text{SO}_4$  were added to the syrup, whereupon crystallization occurred. 4.5 gm. of material which proved to be active acetylproline were obtained. The melting point of a mixture of this material and acetyl-*l*-proline was 116–117° (corrected). The specific rotation of a 0.5 per cent aqueous solution was  $[\alpha]_D^{27} = -107.1^\circ$  which agrees with the value found for the compound when prepared by acetylation in aqueous solution (1).

#### *Analysis*

4.127 mg. substance: 8.16 mg.  $\text{CO}_2$  and 2.52 mg.  $\text{H}_2\text{O}$

3.222 " " : 0.260 cc. N at 31° and 746 mm.

$\text{C}_7\text{H}_{11}\text{O}_3\text{N}$ . Calculated. C 53.46, H 7.05, N 8.91

Found. " 53.92, " 6.83, " 8.89

*Action of Acetic Anhydride on Sodium Hydrocinnamate in Presence of Ethyl Alcohol*—To a solution of 0.240 gm. of sodium in 40 cc. of absolute ethyl alcohol 1.564 gm. of hydrocinnamic acid were added and the mixture was shaken. Sodium hydrocinnamate separated at once and the mixture became almost solid. 5.3 cc. of acetic anhydride were then added and the mixture was stirred

thoroughly until all of the solid had gone into solution. The temperature increased to about 40°. The solution was then allowed to stand, protected from atmospheric moisture by means of a calcium chloride tube, for 5 hours. It was then poured into 150 cc. of water and thoroughly extracted with four 40 cc. portions of ether after 10 cc. of dilute (6 N) sulfuric acid had been added. The combined ether extracts were extracted with 5 per cent sodium carbonate solution so long as a small sample of the aqueous extract gave an oily precipitate when acidified with dilute hydrochloric acid. The combined aqueous extracts were boiled a short time to expel the dissolved ether and most of the alcohol. After the solution had been cooled, it was acidified with dilute hydrochloric acid and then cooled in an ice box. After standing in the ice box overnight the crystals were filtered. The mother liquor was extracted thoroughly with four 25 cc. portions of ether, the ether extract was shaken with a slight excess of 5 per cent sodium carbonate solution, then the ether was distilled. The aqueous residue was worked up in the same way as the original aqueous extract and another appreciable quantity of hydrocinnamic acid was obtained. The combined precipitates were dried in a vacuum desiccator over calcium chloride. The yield was 0.966 gm. or 61.6 per cent of the hydrocinnamic acid originally used. The melting point was 48–48.5°.

The ether layer resulting from the original aqueous extract was dried over sodium sulfate and the ether was fractionally distilled through a 2 foot column. A small volume of an oil was left which was distilled under reduced pressure. It boiled at 94–98° at 9 mm. and weighed 0.6 gm. At atmospheric pressure the liquid boiled at 244° (corrected) by a micro method. Ethyl hydrocinnamate is listed in the literature as boiling at 247–249°. The liquid was refluxed with 5 cc. of 6 N sodium hydroxide until all the oily droplets had disappeared. The solution was then filtered and acidified with dilute hydrochloric acid. After cooling in an ice box and seeding the mixture, crystals formed. They were filtered and dried. The melting point was 46–46.5° and when mixed with known hydrocinnamic acid the melting point was not lowered. The yield of ethyl hydrocinnamate based on the amount of hydrocinnamic acid used was 32 per cent of the theoretical and the yield of hydrocinnamic acid obtained in the saponification of the

ester was 0.369 gm. or 73 per cent of the theoretical. In a preliminary experiment a 26 per cent yield of ethylhydrocinnamate was obtained.

*Acetylmethyl-l-Phenylalanine*—Optically active *N*-methyl-*l*-phenylalanine was prepared by the method of Fischer and Lipschitz (8). The *N*-methyl-*l*-phenylalanine had a specific rotation of  $[\alpha]_D^{31} = +48.9^\circ$  in 0.1 *N* NaOH which is comparable to that of  $+49.74^\circ$ , the value obtained by Fischer and Lipschitz.

0.6 gm. of the material was dissolved in 0.8 cc. of 2 *N* NaOH in 3 cc. of water and the solution cooled in an ice bath. Seven additions of 1.2 cc. of 2 *N* NaOH and 0.1 cc. of acetic anhydride each were made in the usual fashion and the solution was allowed to stand at  $40^\circ$  for 4 hours. After this time, 3.07 cc. of 6 *N*  $H_2SO_4$  were added and the solution reduced to dryness *in vacuo*. The acetylmethylphenylalanine was extracted from the  $Na_2SO_4$  with acetone and the acetone solution reduced to a viscous syrup *in vacuo*. The residue was crystallized from a minimum amount of water. In order to have success in crystallizing the compound, care must be taken to removed all organic solvents. 0.4 gm. of pure material having a melting point of  $144\text{--}145^\circ$  (corrected) and a specific rotation of  $[\alpha]_D^{31} = -76.5^\circ$  (in absolute alcohol) were obtained. The compound crystallized out in tufts of microscopic elongated six-sided platelets.

The acetyl derivative of *dl*-methylphenylalanine prepared from *dl*-methylphenylalanine crystallized from water in diamond-shaped platelets with slightly convex sides and melted at  $146.5\text{--}147^\circ$  (corrected). The *dl*-methylphenylalanine had been prepared by the method of Friedmann and Gutmann (9).

*Attempted Racemization of Acetyl-N-Methyl-l-Phenylalanine*—0.3 gm. of acetyl-*N*-methyl-*l*-phenylalanine was dissolved in 1.4 cc. of 2 *N* NaOH. 3 cc. of water and 1 cc. of acetic anhydride (7.4 mols) were added and the solution was allowed to stand at  $40^\circ$  for 96 hours. 0.93 cc. of 6 *N*  $H_2SO_4$  were added and the material was recovered as above. The specific rotation of the compound had not been affected by this treatment as the recovered material had a rotation of  $[\alpha]_D^{32} = -75.0^\circ$ .

#### *Analysis*

3.275 mg. substance: 0.190 cc. N at  $27^\circ$  and 750 mm.

$C_{12}H_{15}O_2N$ . Calculated, N 6.33; found, N 6.51

The authors wish to thank Professor H. T. Clarke for his helpful criticism of the paper and Dr. J. Harmon for carrying out the experiments on hydrocinnamic acid.

#### SUMMARY

An explanation of the racemization of sodium salts of acylamino acids in aqueous solution by acetic anhydride has been presented upon the basis of a transitory formation of an azlactone ring.

In confirmation of a previous conclusion that amino acids containing a secondary amino group cannot be racemized by this method, it has been shown that the sodium salt of acetyl-N-methyl-l-phenylalanine is not racemized in aqueous solution by acetic anhydride.

The acetylation-esterification reaction of Ashley and Harington has been further investigated. It has been shown that racemization takes place during the reaction, affording further evidence of the theory postulated by them of the temporary formation of the azlactone. Furthermore, it has been shown that proline is not esterified nor racemized under the conditions of this reaction.

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## THE PREPARATION OF SARCOLACTIC ACID

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In spite of the important rôle that optically active sarcolactic acid plays in many fields of biochemistry and physiology, there is no satisfactory method of preparing this acid.

The procedure of Wislicenus (1) is long and results in poor yields. Meat extract is suspended in water and the proteins removed by treating several times with 90 per cent alcohol. The filtrate is evaporated, acidified with sulfuric acid, and extracted six times with ether. The resulting extract is converted into the lead salt. This is decomposed by means of  $H_2S$  and finally a zinc salt is prepared, which must be recrystallized several times to obtain constant rotation. Klimenko (2) improved this method and was able to increase the yield to 1 to 2 per cent, calculated on the basis of meat extract employed.

Jungfleisch and Godchot's (3) method of separating the lactic acid antipodes by means of the quinine salt yields only the levorotatory form. It is almost impossible to obtain sarcolactic acid or its salts. Only partial separation is possible when *dl*-lactic acid is resolved into its optically active components by means of the difference in solubility of their morphine salts in water.

An interesting paper by Kimura (4) reports the formation of optically active lactic acid when glucosamine or *dl*-lactic acid is fermented by *Bacillus prodigiosus* and *Bacillus subtilis*. The amounts of sarcolactic acid isolated are, however, extremely small.

During an investigation<sup>1</sup> on the constitutional relationship of lactic acid and alanine, it was necessary to obtain large quantities of sarcolactic acid. The new method described is so simple that

<sup>1</sup> Carried out, in 1928, in the Department of Chemistry, University of Heidelberg. The writer is indebted to Professor Karl Freudenberg for valuable criticism and advice.

by its use sarcolactic acid should be made readily available for physiological and chemical investigations.

#### EXPERIMENTAL

200 gm. of Liebig's meat extract were suspended in 1000 cc. of water and acidified with sulfuric acid until a sample gave a distinct acid reaction with Congo red. The liquid was then transferred to a continuous liquid extractor and extracted for 3 days with ether. The extractor should be large enough so that the foam from the solution to be extracted will not be carried over with the ether into the receiver. A smaller extractor may be used if a piece of fine metal gauze is placed in the upper part of the extractor to break up the foam. Ethyl acetate instead of ether is equally satisfactory as a solvent. The best yields were obtained when this extraction was performed in a vacuum at 25–30°.

The solvent was evaporated from the extract and the residue dissolved in water, acidified, and again extracted for 2 days in the same manner. This final product, completely freed from the solvent *in vacuo*, weighed 12 gm. Distillation in a high vacuum gave 8 to 10 gm. of a sarcolactic acid boiling under 0.2 mm. at 98–100°.

Racemization into inactive anhydro forms which according to Wislicenus (1) takes place at 135–150° under normal pressure is thus prevented.

Sarcolactic acid prepared in this manner is very pure as isolated. The zinc salt after being recrystallized once from hot water showed a rotation of  $[\alpha]_D^{18} = -7.3^\circ$ ; the zinc ammonium salt,  $[\alpha]_D^{18} = -10.8^\circ$ .

#### SUMMARY

A simple method is reported for the preparation of sarcolactic acid in pure form. Yields of 4 to 5 per cent are easily obtained.

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# CHEMICAL STUDIES ON A PARATHYROID HORMONE\*

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## INTRODUCTION

During the past 10 years it has been generally accepted that a protein fraction containing a blood calcium-raising principle may be extracted from parathyroid glands. Methods of extraction and fractionation have been described by Hanson (1), Berman (2), Collip (3), Fisher and Larson (4), Hjort, Robison, and Tendick (5), Davies, Dickens, and Dodds (6), and Tweedy (7-9).

In 1925, Collip and Clark (10) described some of the physical and chemical properties of one of their hormone preparations, and state, "The physical and chemical properties of this substance are in many ways similar to those of insulin." Freudenberg and Dirscherl (11) have adopted the view from their studies on amorphous and crystalline insulin that the hormone forms only a small portion of the insulin molecule, and is probably incorporated therein by peptide linkage. Furthermore, these authors are of the opinion that in the study of parathyroid hormone, pepsin, and urease one is confronted with a similar problem.

Collip (12), Allardyce (13), and Tweedy and Smullen (8), in further attempts to concentrate the active principle in their extracts, have attempted to apply the methods developed by Abel and coworkers (14) and by Harington and associates (15)

\* Abstracted, in part, from a dissertation submitted by Masamichi Torigoe to the Graduate School of Loyola University in partial fulfillment of the requirements for the degree of Master of Science. A portion of the results given here was presented before the local Biochemical Group of the Chicago Section of the American Chemical Society, March 18, 1932, and also before the American Society of Biological Chemists at Philadelphia, April 27, 1932.

for the purification and crystallization of insulin. Neither by these methods, nor by various modifications of them, has a crystalline product been obtained.

Inasmuch as attempts to crystallize the active protein have been unsuccessful, it has appeared worth while to continue the studies of the effects of various reagents on the hormone in the hope that the presence of specific chemical groups, identified with its physiological activity, might be revealed. It was also hoped that such information might contribute toward the ultimate development of a method of separating the active principle from the protein complex.

Evidence is presented in this paper of inactivation, and reactivation of parathyroid hormone, which strongly suggests that the  $\text{NH}_2$  or  $\text{NH}$  group, or groups, and possibly the  $\text{COOH}$  group, or groups, are of importance in the physiological action of parathyroid hormone.

#### EXPERIMENTAL

##### *Method of Biological Assay*

The hormone preparations used in these studies were prepared by the methods developed by one of us (7-9), and were standardized by the procedure of Collip and Clark (16). Accordingly, 100 units of the hormone preparation constitute an amount sufficient to increase the blood plasma calcium of a dog weighing 20 kilos 5 mg. per cent over a period of 15 hours. Twelve to fifteen different dogs were used in the standardization of each of the two preparations used in these studies, and over 200 different dogs were used in connection with the potency tests in the experiments that follow. An increase in the total plasma calcium of 0.5 mg. per cent, or less, was not interpreted as due to injected hormone, but rather to physiological variation, combined perhaps with analytical error. Calcium analyses were made by the Kramer and Tisdall method (17), as modified by Tweedy and Koch (18).

##### *Effect of Formaldehyde*

Since the senior author (W. R. T.) had not found it possible to ascribe the variability in potency of certain of his preparations to a variation in the nitrogen partition as determined by the

Thimann procedure (19), it became a matter of interest to determine whether a nitrogen-containing group is concerned in the hormone activity. The success of Freudenberg and coworkers (20) in reactivating insulin, inactivated by formaldehyde, suggested to us the feasibility of making a similar study on parathyroid hormone.

*Inactivation*—Preliminary experiments were carried out in which the concentration of hormone and formaldehyde, the time of contact, and the temperature were varied. Only the final procedure as adopted for complete inactivation is recorded in detail.

1 gm. (2000 units) of the parathyroid hormone preparation was suspended in 30 cc. of 40 per cent commercial formaldehyde,<sup>1</sup> and adjusted to a pH of 4.6. The mixture was allowed to remain at room temperature for a period of 2 hours, after which absolute alcohol was added sufficient to give a concentration of 75 per cent. 2 volumes of anhydrous ether were then added, and an hour at 0–2° allowed for precipitation of the dissolved material. The insoluble material, recovered by centrifugation, was then washed several times with ether, after which it was dried *in vacuo* at room temperature. The product obtained was insoluble in water and constituted from 80 to 85 per cent of the starting material.

The comparative potency tests on the inactivated hormone, and its reactivated form, are shown in Table I. The “expected” increment in blood plasma calcium per 100 cc. has been arrived at by the following calculation,  $\frac{20 \times 5 \times \text{units of dose}}{\text{weight of test dog in kilos} \times 100}$ ,

where 20 is the weight in kilos of the standard dog, and 5 is the calcium increment for 100 units of standard preparation. Huge doses, as measured in units of the standard preparation, have been used in order that the certainty of complete inactivation might be more clearly established. The fact that one dog in each of the two designated groups (Table I) did not react to the hormone is attributable to the wide variation sometimes found in the reactivity of different dogs to comparable amounts of the hormone.

It appears that inactivation can safely be attributed to the binding by formaldehyde of a nitrogen-containing group—most probably an  $\text{NH}_2$  or an  $\text{NH}$  group, or groups—which may be essential

<sup>1</sup> The formaldehyde content was found to be 40.6 per cent by the iodometric method.

to the physiological action of the hormone. We feel positive that the hormone preparation is not inactive because of slow absorbability, since a water-insoluble product, produced by precipitation of the original untreated hormone preparation from a 0.4 per cent acetic acid solution, remained highly potent.

*Reactivation*—The first demonstrable reactivation was produced by using essentially the same method adopted by Freudenberg and coworkers (20) for the reactivation of formaldehyde-inactivated insulin.

TABLE I

*Comparative Potencies of Formaldehyde-Inactivated Parathyroid Hormone and the Reactivated Product*

Product No	Dogs used	Average weight	Average dose	Blood plasma calcium increment	
				Expected*	Average obtained
		kg	units†	mg per 100 cc.	mg per 100 cc
1-6. Inactivated	6	16.9	101	6	0.15
7. " "	1	12.5	100	8	0.15
7. Reactivated	4	11.8	95	8	3.51
8. Inactivated	1	13.9	111	8	-0.50
8. Reactivated	8*	11.3	91	8	1.84
9. Inactivated	1	12.5	115	9	-0.69
9. Reactivated	6*	16.5	95	6	1.52
10. Inactivated	2	14.5	110	7	0.17
10. Reactivated	3	15.1	124	8	3.35
11. Inactivated	1	10.0	90	9	-0.35
11. Reactivated	3	17.1	126	8	4.62

\* Discussed in text statement.

† 1 gm. = 2000 units.

100 to 200 mg. of tested inactivated hormone (Table I) were suspended in 10 cc. of 0.001 N hydrochloric acid, and boiled on a water bath for 20 minutes. After about 15 minutes, the suspended material rapidly dissolved, producing an amber-colored solution which was found to contain the hormone in partially reactivated form (Table I).

Control experiments on the original untreated hormone preparation have shown that no activity is lost, or produced, when it is

treated by the above procedure. It should also be added that in additional experiments it has been found unnecessary to use added acid in the reactivation of hormone inactivated in acid formaldehyde, since enough remains firmly bound to the recovered product to reactivate it, when it is boiled in water for a few minutes. It is only when inactivation is accomplished in an alkaline formaldehyde solution that added acid is necessary for reactivation.

#### *Effect of Acid-Ethyl Alcohol*

In a previous publication (9) it was reported that parathyroid hormone is inactivated when suspended in absolute ethyl alcohol containing 0.5 per cent HCl, and heated for 20 minutes at 70°; or, when suspended for 1 hour at 10° in absolute ethyl alcohol saturated with dry HCl gas. Attempts to reactivate the hormone by contact with dilute alkali were unsuccessful.

Recently, we have found that complete inactivation is produced by 0.75 N HCl in 75 per cent ethyl alcohol after a period of 24 hours at room temperature. The same inactivating mixture was used by Carr and coworkers (21) in the inactivation of insulin, which they subsequently partially reactivated by contact with N/11.875 sodium hydroxide at 0° for 17 hours. Similar treatment has not resulted in reactivation of our product. Control experiments on the untreated hormone preparation have shown that the maximum strength of alkali consistent with retention of activity, yet favorable to ester hydrolysis, was employed. We are, therefore, inclined to believe that inactivation was produced by some reaction other than, or additional to, esterification.

#### *Effect of Acid-Methyl Alcohol*

Partial reactivation was accomplished when hormone preparations inactivated by acid-methyl alcohol were used. The potency tests on the inactivated product are shown in Table II. The procedure used was practically the same as that used by Charles and Scott (22) in similar experiments on insulin, and is as follows:

750 mg. of the hormone preparation were suspended in each 20 cc. portion of 0.1 N HCl in absolute methyl alcohol,<sup>2</sup> and allowed to remain in a tightly stoppered 50 cc. centrifuge tube at room tem-

<sup>2</sup> Redistilled over calcium oxide.

perature for a period of 24 hours. An excess of acetone was added, and after 1 hour the insoluble material, separated by centrifugation, was washed several times with acetone and dried *in vacuo*.<sup>3</sup> The remaining material, which largely precipitated after several days, was found to be inactive, but could be reactivated as easily as the first portion recovered. It should be added that it is to be expected that the conditions for complete inactivation by this method will probably be found to vary, depending upon the purity of the hormone preparation used.

It has been observed that, accompanying inactivation of parathyroid hormone, there occurs a shift in the isoelectric point from pH 5.8 to pH 8.2.<sup>4</sup> No appreciable shift in the reverse direction is produced as a result of reactivation attained in our experiments.

Carr and coworkers (21) have interpreted the reversible shift in the isoelectric point of insulin under these conditions as evidence of ester formation, while Charles and Scott (22) suggest that it may be due to the attachment of the alkyl group to the amino group. Jensen (24) has suggested that ester formation may be followed by the formation of a diketopiperazine derivative.

Whether parathyroid hormone inactivation is induced through either, or both, alkylation and esterification remains to be shown.

*Reactivation*—Preliminary experiments carried out on the stability of the original untreated hormone to alkali demonstrated that contact with 0.08 N sodium hydroxide could be maintained for 30 hours at 0–2° without appreciable loss in activity. Furthermore, it was found that weaker alkali was ineffectual in the restoration of activity after acid-alcohol inactivation. The procedure below was used in the reactivation of the previously tested inactivated products shown in Table II.

3 cc. of cold 0.08 N sodium hydroxide were added to each 100 mg. of the product to be reactivated. The material was stirred from time to time, and allowed to remain at 0–2° for 30 hours. The

<sup>3</sup> In connection with a separate investigation, McJunkin, Tweedy, and Breuhaus (23) have described the comparative effects of excessive doses of the hormone and its acid-alcohol-inactivated form on the tissues of the rat.

<sup>4</sup> The technique employed by Carr and coworkers (21) was used in these determinations.

pH of the brownish colored solution was adjusted to near neutrality and immediately tested for potency (Table II).

In each of the two designated groups of animals (Table II) there were two animals in which no increase in blood calcium was detected. The amount of reactivation, although positive, was insufficient to produce a noticeable mobilization of calcium in the blood of these four dogs.

TABLE II  
*Comparative Potencies of Acid-Methyl Alcohol-Inactivated Hormone and the Reactivated Product*

Product No.	Dogs used	Average weight	Average dose	Blood plasma calcium increment	
				Expected*	Average obtained
		kg.	units†	mg. per 100 cc.	mg. per 100 cc.
7. Inactivated	2	17	108	6	0.34
7. Reactivated	2	17	128	7.5	2.78
8. Inactivated	2	17	128	7.5	0.04
8. Reactivated	3*	16	120	7.5	1.19
9. Inactivated	5	16	120	7.5	0.46
9. Reactivated	6*	17	125	7.5	1.80
10. Inactivated	5	11	83	7.5	0.11
10. Reactivated	1	10	75	7.5	1.09

\* Discussed in the text statement.

† 1 gm. = 2000 units.

### *Effect of Nitrous Acid*

While analyzing one of our preparations for amino acid nitrogen by the Van Slyke procedure, the observation was made that the deaminized product assumed a yellow color. A potency test on the recovered product indicated that complete inactivation was produced coincident with the removal of the total amino nitrogen, which was found to constitute 6 per cent of the total nitrogen of the product. Recently several additional potency tests have been made on deaminized products, prepared by essentially the same method (modification of Skraup's method) used by Dunn and Lewis (25), which seeks to avoid a possible slight hydrolytic action of nitrous acid.

750 mg. (1500 units) were dissolved in 50 cc. of water, and 5 cc. of glacial acetic acid were added dropwise, followed by the dropwise addition of 10 cc. of 10 per cent sodium nitrite. A yellow precipitate gradually settled out over a period of 18 hours. After neutralization, and suitable dilution, the product was tested for potency. The blood plasma calcium of five dogs (average weight 14 kilos) injected with a dose averaging 141 units (original unitage) was not increased above its normal value.

Our present information does not warrant the conclusion that deamination alone was responsible for inactivation. The effects produced by the action of nitrous acid on the hormone may be manifold. Dunn and Lewis (25) suggest that the yellow color of deaminized proteins may be due to the formation of nitroso compounds, resulting from the nitrosation of imino nitrogen in the amino acids, histidine, tryptophane, arginine, and proline; and that nitrosation of tyrosine may occur in the position ortho to the hydroxyl group. The possibility that inactivation of parathyroid hormone may have been due to a structural alteration of the above character, rather than to oxidation or deamination must be recognized.

#### *Adsorption on Permutit*

In connection with the above studies, one of a series of adsorption experiments may be cited as evidence of basic nitrogen groups in the active protein. Whitehorn (26) has found that nitrogen bases with a dissociation constant of  $5 \times 10^{-9}$ , or over, are quantitatively adsorbed on activated permutit, while weaker bases, and non-basic substances, are not. We reasoned that if the active principle was loosely associated with a protein of sufficient basicity, or was itself a basic or non-basic substance, then purification might be effected by means of permutit.

Our experiments have demonstrated that the active protein may be adsorbed by permutit from acid solution at various pH values, and that it can be released from permutit by means of 5 per cent ammonium hydroxide at 0-2°. Saturated potassium chloride solution, 90 per cent carbolic acid, 50 per cent aqueous pyridine, and 5 per cent ammonium chloride were found ineffective as release reagents.

Repeated potency tests have shown that no increase in activity

was effected by adsorption on, and release from permutit. These experiments are of interest, however, as further emphasizing the intimate relationship of the active principle to protein from which it has not been separated. It may be an integral part of a protein molecule, or it may be that the simultaneous adsorption, and release, of active principle and protein occurred, and hence no change in activity was detected.

#### SUMMARY AND CONCLUSIONS

1. Parathyroid hormone is completely inactivated by a 40 per cent aqueous solution of formaldehyde within 2 hours at room temperature. The resultant product may be partially reactivated by boiling in a very dilute acid solution for a period of 20 minutes. These results indicate that either, or both, the  $\text{NH}_2$  and the  $\text{NH}$  group, or groups, is necessary to the physiological action of parathyroid hormone.

2. Parathyroid hormone, which has been completely inactivated by acid-ethyl alcohol, has not been reactivated by dilute alkali. Complete inactivation by acid-methyl alcohol gives a product which can be partially reactivated by contact with 0.08 N alkali at 0–2° for 30 hours.

3. Complete inactivation by nitrous acid indicates, but does not prove, that a nitrogen-containing group (amino or imino), or groups, is necessary to the physiological action of the hormone.

4. The active protein has been adsorbed on activated permutit, and released therefrom by 5 per cent ammonium hydroxide at 0–2°.

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## STUDIES ON VITAMIN G (B<sub>2</sub>) WITH SPECIAL REFERENCE TO PROTEIN INTAKE

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While the investigations of vitamin G carried on in this laboratory are planned with primary reference to normal nutrition rather than to the pellagra problem as such, the literature of pellagra suggested to us the need of a study of the relationship of protein intake to the phenomena of vitamin G deficiency.

In 1925, Goldberger and Tanner (1) reported that an adequate supply of washed casein helped to prevent or ameliorate human pellagra; and a later paper (2) from the same laboratory mentioned casein as apparently beneficial in the treatment of black tongue in dogs. But in most of the recent writings of the Goldberger school, as well as of several other investigators, human pellagra, black tongue of dogs, and the so called pellagra-like condition of experimental rats, all seem to be discussed in terms of vitamin deficiency, with practically no apparent reference to protein.

As the earlier experimental indications of some relation of protein to this general problem seemed too suggestive to be set aside entirely, we were led to undertake the experiments here reported in which different levels of protein intake were tested as to their influence upon the development of the so called pellagra-like condition in rats whose diets were more or less deficient in vitamin G (B<sub>2</sub>). In accordance with our understanding of present usage, the latter term is here applied to the more heat-stable part of the vitamin B complex without prejudice to the further problem of the possible multiple nature of this nutritional factor.

### EXPERIMENTAL

*Experimental Diets*—The composition of the experimental diets here used is shown in Table I. The sources of vitamin G (B<sub>2</sub>)

and of protein, as bearing especially upon the present problem, are discussed briefly in the sections immediately following. Regarding the other components of the diet it seems sufficient to point out that they represent not only adequate but liberal provision for all other known nutritional needs of the experimental animals. Guided by the experience of Bourquin in this laboratory (3), we provided the vitamin B ( $B_1$ ) of these diets as follows:

2½ kilos of ground whole wheat were shaken for 1½ hours with 4.44 liters of 84.5 per cent alcohol.<sup>1</sup> The extract was filtered through a Buchner funnel and the residue was suspended in 3.13

TABLE I  
*Composition of Experimental Diets*

Diet No	Series I			Series II		
	570	571	572	575	576	577
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Total protein ( $N \times 6.25$ )	6	12	18	6	12	18
Activated corn-starch (wheat extract)	20 1	20 1	20 1	20 1	20 1	20 1
Osborne and Mendel salt mixture*	4	4	4	4	4	4
Butter fat, filtered	9	9	9	9	9	9
Cod liver oil	1	1	1	1	1	1
Agar	2	2	2	2	2	2
Skim milk powder				3 3	3 3	3 3
Casein, extracted with 60 per cent alcohol	6 8	14	21 3	5 4	12 6	19 9
Corn-starch	57 1	49 9	42 6	55 2	48 0	40 7

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919)

liters of 80 per cent alcohol and shaken for 1 hour, filtered, and the residue washed with 0.95 liter of 80 per cent alcohol and pressed as dry as possible. The combined extracts and washing were concentrated to one-fourth their volume under reduced pressure at a temperature of 28-32°. The concentrated extract was poured upon 937 gm. of corn-starch and dried before an electric fan at

<sup>1</sup> The strength (concentration) of alcohol is stated by weight, and the amounts used are such as to insure the presence of 80 per cent alcohol after the slight dilution of the solvent by the hygroscopic moisture of the ground wheat.

room temperature. This dried material was incorporated in the diet in such quantity that 1 kilo of diet contained the extract from 500 gm. of ground whole wheat. This proportion has been shown by Bourquin and others to be sufficient to supply fully the rat's need of vitamin B ( $B_1$ ) for growth.

*Sources of Vitamin G ( $B_2$ )*—As in all experiments with rats of good nutritional history, one source of vitamin G ( $B_2$ ) is the bodily store possessed by the animal at the beginning of the experiment. In Series I, of the experiments here described, the only other source (so far as known) was the very small amount of vitamin G which was contained in the strong alcohol extract of whole wheat here used as source of vitamin B ( $B_1$ ). This would appear, from previous experience of this laboratory, to be almost negligible, so that the animals of Series I were probably chiefly dependent during the experimental period upon the vitamin G which they had previously stored. In Series II, there was an appreciable daily intake of this vitamin, supplied by the presence of 3.3 per cent of skim milk powder in the diet. The purpose here was to test the effect of varied protein intake when vitamin G intake was significantly appreciable, though not adequate for the continued support of normal growth and health. In an additional control series an abundance of vitamin G was supplied at the lowest of the three levels of protein intake.

*Sources of Protein*—The very small amount of alcohol-soluble protein introduced with the vitamin B ( $B_1$ ) of all the experimental diets was constant throughout. In Series I, casein (previously extracted with 60 per cent<sup>2</sup> alcohol) was practically the sole significant source of protein supplied. The air-dry casein was analyzed and incorporated in the diets in such proportions that the three diets (Diets 570, 571, 572) contained 6, 12, and 18 per cent protein, respectively. In Series II, the natural protein mixture of 3.3 per cent of skim milk powder was a constant to which was added enough of the extracted casein to bring the total protein content of these three diets (Diets 575, 576, 577) to 6, 12, and 18 per cent, respectively.

*Experimental Animals and Their Caging*—Young albino rats from families whose food consisted mainly of a mixture of dried

<sup>2</sup> Throughout this work, concentration of alcohol is indicated as percentage by weight.

whole milk and ground whole wheat were separated at 28 to 32 days of age and placed in individual cages with raised wide meshed screen bottoms to minimize the possible effects of coprophagy. Weighed portions of the experimental diets were supplied in such quantities that the animals could consume the diet *ad libitum* while at the same time the food intake was accurately known by weekly periods. Fresh distilled water was at all times available to the experimental animals.

Approximately equal numbers of male and female animals were placed on each different experimental diet; and the experimental animals (all of which had been bred in the laboratory) were carefully matched by threes for distribution among the three diets which in each series were compared in strict parallel. The experimental animals were under the daily observation of one of us (I. A. D.).

#### DISCUSSION

Series I included 96 experimental animals on diets markedly and equally deficient in vitamin G; but divided as explained above into three divisions receiving respectively 6, 12, and 18 per cent of protein. Under these conditions and within these limits, the average rate of growth increased with the protein intake, the difference in results between 6 and 12 per cent being much greater than that between 12 and 18 per cent.

Series II included 65 experimental animals on diets less markedly deficient in vitamin G and again distributed over the same three quantitative levels of protein intake. Here also the rate of growth increased with the level of protein intake, and the difference in effects between 6 and 12 per cent was greater than that between 12 and 18 per cent.

An additional control series of twenty-four experimental animals received a diet containing 6 per cent protein and made up in the same manner as the diets of Series I except that there was added a liberal supply of vitamin G in the form of protein-free milk made essentially as described by Osborne and Mendel (4). Although the amount of protein and amino acids thus introduced was too small to have any appreciable effect (0.01 gm. of nitrogen per rat per week) and moreover was of a kind contained in larger amounts in all the diets of Series II, yet these animals grew at a

much higher rate than any of the others. Hence it is clear that the checking of growth was not due in any of these cases to low protein intake in itself. Yet while the checking of growth was primarily (or essentially) due to shortage of vitamin G, the extent of the effect was markedly influenced by the protein intake.

The weight data are briefly summarized in Table II.

TABLE II  
*Summary of Weight Data of Experimental Animals (Rats)*

		No. of cases	Mean gain in 8 wks.	Probable error of mean
			<i>gm.</i>	
Series I				
Group A.*	6 per cent protein..	18	-2.6	±0.7
	12 " " " ..	17	10.6	±1.1
	18 " " " ..	18	14.5	±1.3
Group B.	6 " " " ..	15	-1.5	±0.8
	12 " " " ..	12	8.4	±1.3
	18 " " " ..	16	11.2	±1.0
Series II				
Group A.*	6 " " " ..	11	10.9	±1.0
	12 " " " ..	10	21.1	±2.0
	18 " " " ..	11	32.6	±1.9
Group B.	6 " " " ..	11	8.9	±1.2
	12 " " " ..	10	22.0	±2.0
	18 " " " ..	12	28.0	±1.2
Additional control series				
Group A*.....		12	60.3	±1.2
" B.....		12	57.7	±1.4

\* Animals of Group A were from preexperimental diets somewhat lower in vitamin G than were those of group B.

That vitamin G is the first limiting factor in all of the diets of Series I and II is further evidenced by the fact that the symptoms (other than checking of growth) which developed on these diets were those which have been seen in other cases of shortage of vitamin G and which were absent in the cases, carefully studied by Woods (5), in which growth was checked by shortage of cystine induced by extreme reduction of intake of dietary protein of the same character as was employed in the present experiments.

Thus there is no room for doubt that the nutritional deficiency

with which we are here dealing is essentially the so called pellagra-like condition resulting from shortage of vitamin B<sub>2</sub> or G as described by the research workers of several different laboratories, yet both the checking of growth and the development of the pellagra-like symptoms were influenced by the protein intake.

The variability of the outward symptoms of the vitamin G avitaminosis has been discussed in previous papers from this laboratory (6). This may be in part a strictly individual variability, in part seasonal, and in part due to differences in the nutritional backgrounds of the experimental animals in different laboratories or in the same laboratory at different times. In the work here recorded, the first symptom to appear was a hemorrhagic condition in which a small amount of blood, coming from the nose, was usually first seen upon the paws. The characteristic dermatitis appeared somewhat later, most frequently on the backs of the paws, the shins and thighs, and the back and shoulders. Provided the animal had not been allowed to lose too greatly its general physical vigor, the skin symptoms could be cleared up and rapid gain of weight induced by supplementing the diet with protein-free milk. The animals frequently showed a loss of hair in bilaterally symmetrical areas on the shoulders and back and around the eyes. This condition was more pronounced in the case of the animals receiving only 6 per cent protein in the diet. Upon autopsy most of the animals which had shown pronounced outward symptoms were found to have developed an unhealthy condition of the intestines with bloody mucus and varying degrees of disintegration of the mucous membranes of the digestive tract, often foreshadowed in the period just before death by bloody evacuations from the intestines.

The length of time of survival of the experimental animals was also influenced by the protein intake. Thus in Series I none of the animals receiving 6 per cent protein survived beyond 20 weeks, whereas 30 per cent of those receiving 12 per cent protein and 44 per cent of those receiving 18 per cent protein survived beyond this period.

#### SUMMARY

Experimental animals which received diets of liberal protein content (12 or 18 per cent) were less severely affected by shortage

of vitamin G ( $B_2$ ) than were the strictly comparable animals which received protein of the same kind at a much lower level (6 per cent of the diet), even though the protein was of excellent nutritive value.

In so far as the symptoms of shortage of vitamin G are regarded as pellagra-like, such a vitamin theory of the disease should not preclude recognition of the possibility that the protein supply may also have a significant bearing upon the pellagra problem.

And in so far as the pellagra problem is to be regarded as nutritional, one should think not in terms of a choice between "protein theory" and "vitamin theory," but rather of a theory broad enough to take account of the possible participation of more than one nutritional factor.

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# THE SOLUBILITY OF THE PLASMA PROTEINS

## I. DEPENDENCE ON SALT AND PLASMA CONCENTRATIONS IN CONCENTRATED SOLUTIONS OF POTASSIUM PHOSPHATE\*

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### INTRODUCTION

Since Panum (1) in 1851 found that protein separated from serum on dilution and the addition of acid, the most widely used means of defining the plasma proteins has been by describing their solubility behavior. Between 1859 and 1902 the work of Denis (2), Hammarsten (3), Burekhardt (4), Marcus (5), Kauder (6), Pick (7), and others established the definitions of these proteins as commonly used today. These definitions are based on studies of the solubilities of so called protein fractions separated from plasma or serum by repeated precipitations and re-solution under specified conditions. The terms fibrinogen, euglobulin, pseudoglobulin, and albumin, if strictly used, should, therefore, designate the proteins separated by such procedures.

Between 1890 and 1905 various attempts were made to measure the concentration of the individual proteins of serum by so called single fractional precipitation; that is, by determinations carried out on precipitates or filtrates from the first precipitation at specified salt concentrations.

In 1901 Pinkus (8) published a method for estimating the serum proteins by such fractional precipitation with sodium sulfate at 37°. His method assumed no overlapping of the precipitation

\* This work was made possible by the generous contributions of the Joan McLeod Miller Fund of the Department of Pediatrics, Harvard Medical School.

zones. There was, however, no attempt to analyze the purity of the fractions. Porges and Spiro (9) in 1903 studied such fractional precipitation of serum proteins with ammonium sulfate and found that the concentration of salt solution required to precipitate completely a less soluble fraction extended beyond the concentration at which the more soluble fraction commenced to precipitate out. They state that dilution of serum permits more complete separation, but analyses adequately supporting this claim are not given.

Haslam (10, 11) in investigating the separation of serum proteins by fractional precipitation demonstrated the incompleteness of separations effected by this method. He showed that dilution of serum before precipitation resulted in less of the more soluble protein being carried down in the precipitate of the less soluble protein, and that with sufficient dilution such inclusion caused only a small error. But he also demonstrated that dilution would not lessen the relative concentration of the less soluble protein remaining in solution in the filtrate of the more soluble fraction. The solubility of the less soluble protein in the presence of the soluble protein in salt concentrations at which the latter commenced to be precipitated was such at dilutions of 1:8 or more, that as much as 25 per cent of the euglobulin in ox serum was found in the pseudoglobulin filtrate, and some euglobulin was always found in the albumin filtrate. He interpreted his results as indicating quantitatively the degree of error involved in separations of this kind. If his method of analysis is accepted as applicable to the estimation of errors of single fractional precipitation, his results indicate that the actual solubilities of the less soluble fractions at specified salt concentrations are sufficiently large to vitiate results for the concentrations of the individual protein fractions based on zero solubility of the less soluble fractions at such salt concentrations.

Wiener (12) in 1911 demonstrated the same finding as Haslam concerning the increase in purity of the precipitate as a result of dilution. Apparently unaware of Haslam's finding concerning the amount of the less soluble protein remaining in the filtrate as a result of dilution, Wiener advocated dilution of serum as a means of increasing the accuracy of fractional precipitation.

Haslam's work (11) called attention to a further problem to be

considered in salting out procedures. He observed that purification of the globulin fractions beyond a certain point caused unmistakable changes in the properties of the protein fractions. Chick (13) observed similar changes. Thus arose two questions: are the individual protein fractions separable by salting out procedures distinct individual proteins, and are the fractions so separated similar to the protein complexes existing in plasma?

Chick (13) believed that euglobulin was a complex of pseudoglobulin and a phosphorus-containing serum lipid. Analysis of the amino acid composition of the three purified serum protein fractions by Hartley (14) showed no difference in the amino acid composition of euglobulin and pseudoglobulin, but did reveal a difference between globulin and albumin. Woodman (15) in studying the identity of related proteins found no evidence that serum euglobulin and pseudoglobulin were different.

Svedberg and Sjögren (16) concluded from their experiments on the molecular weights of the serum proteins that there are but two proteins in serum, *i.e.* globulin and albumin. They believed that decomposition of the proteins occurs during such salting out procedures as used by Sørensen (17), and they regarded euglobulin and pseudoglobulin as artificial products.

Sørensen (18) has discussed at length both parts of the question raised. He agrees with Svedberg and Sjögren that the changes in the composition of the protein solutions necessitated by salting out procedures alter the composition of the protein complexes. But he makes a sharp distinction between reversible dissociation changes and irreversible decomposition or denaturation. He considers the serum proteins as complexes existing in serum in reversibly dissociable component systems. "Within each complex all the atom groups are interlinked by main valencies, whereas the components are reversibly linked by means of residual valencies." It is such reversible changes that occur during the proper fractionation of the serum proteins to euglobulin, pseudoglobulin, and albumin. Indeed any attempt to separate the proteins from serum probably alters the protein component systems and in all probability the fractions separated out will differ with the procedure used and will be different from the component systems existing in serum.

This brief and incomplete review gives in outline the background

on which salting out methods for the determination of the concentration of the serum proteins, such as that of Howe (19), should be considered and indicates the care with which results so determined should be interpreted.

Cohn (20), in 1925, showed that the solubility of a protein in concentrated salt solution was related to the concentration of the salt according to the equation,  $\log S = \beta - K_s\mu$ , where  $\beta$  is a constant related to the point of interception between the straight line and the ordinate, where  $\mu$  represents the concentration of salt, in this particular case as represented by the ionic strength, but equally well expressed in terms of molar concentration, and where  $K_s$  is a constant representing the slope of the curve and has been shown to be a characteristic of any individual protein for a given salt solution. In 1928, Cohn and Green (21) showed the relationship between this equation and that of Debye and Hückel expressing the solubility of a saturating body in a solution of a strong electrolyte. Cohn's equation shows that the solubility of a protein plotted against increasing concentration of salt solution results in a logarithmic curve or that the plotting of the logarithm of the solubility against the increasing salt concentration results in a straight line, whose slope,  $K_s$ , is specific for an individual protein in a given salt solution, and hence is an added means of defining a protein.

If one examines a solubility curve of plasma or serum as presented by Howe (19) or Ruszczynski (22), it is apparent that the applicability of a linear relation between the logarithm of protein solubility and salt concentration to each portion of the curve would permit an estimation of the solubility of the less soluble fraction at points of break in the curve and hence at the salt concentrations selected for fractional precipitation. Mâcheboeuf, Sørensen, and Sørensen (23) have shown that such a linear relation exists for preparations of egg albumin which do not behave strictly as a pure protein; that is, whose solubility is not independent of the amount of protein used in the experiment. And Sørensen (18) has found a similar relation in experiments on serum albumin in which the dissociation tendency and dependence of solubility on total protein in the experiment is greater than for egg albumin. It, therefore, seemed to us that this linear relation might be found to hold for the different portions of precipitation curves of plasma.

So far as we have been able to ascertain the existing data on precipitation curves are unsatisfactory for such an analysis. We have, therefore, for the purpose of supplying such data undertaken a series of experiments on the solubility of the plasma proteins.

Cohn (24) has presented data concerning the variation of the ionic strength of potassium phosphate solutions with the maintenance of constant pH, and *vice versa*. The data make this salt particularly suitable for salting out experiments, as its use enables one readily to vary independently pH, salt concentration, serum concentration, and temperature and thus express the solubility of the saturating body as a function of any one of these variables. Florkin (25) and Green (26) have demonstrated the advantages of such phosphate solutions in protein solubility studies.

### *Outline of Experiments*

The experiments reported in this paper deal primarily with the relation between the solubility of the protein complexes of horse and human plasmas and the salt concentration in concentrated potassium phosphate solutions of a constant pH of 6.5 and temperature of 25°.¹ Two experiments pertinent to this study in which the plasma concentrations were altered are included. These two experiments present data concerning the relation between plasma concentration and protein solubility.

In spite of the fact that most solubility studies on the plasma proteins have been carried out in ammonium sulfate and sodium sulfate solutions and that the use of a new salt makes the solubility data less comparable, we selected potassium phosphate for the precipitating reagent in our experiments because of the advantages indicated above.

The pH of 6.5 was determined by using phosphate solutions consisting of equimolecular amounts of dibasic and monobasic potassium phosphate and was selected merely as a matter of convenience, as dilutions could be made from a 3 molal solution²

¹ Experiment 1 was an exception as this was carried out at a temperature of 20°.

² This solution was made up in 2 liter lots as follows:  $\text{KH}_2\text{PO}_4$  is ground in a mortar and dried. From this 817 gm. of salt are accurately weighed out and poured dry into a 2 liter volumetric flask. Any salt adhering to the beaker in which the weighing was carried out is dissolved and washed quan-

with the constant pH remaining in all dilutions. 0.5 cc. of plasma<sup>3</sup> was added to 15 cc. of phosphate solution in order to provide a large dilution of the plasma so that the effect of the total protein or the individual constituents of serum upon the solubility of the individual proteins would be reduced as much as possible, and also so that the inclusion of a more soluble fraction in a less soluble precipitate would be minimal. The salt concentration to which the plasma was added varied from 0.6 molar to 3.0 molar. A period of at least 12 hours was allowed for equilibrium to be reached,<sup>4</sup> during which time the solutions immersed in a constant temperature water bath at 25° were gently shaken by a mechanical shaker. The solutions were then filtered at 25°, precautions being observed to prevent evaporation and filtrations being repeated until the filtrate was clear. The nitrogen in the filtrates was determined by Kjeldahl's macro method.

#### *Experimental Solubility Curves*

In describing the solubility curves we have found it convenient to identify the several portions of the curves with the protein fractions, *i.e.* fibrinogen, euglobulin, pseudoglobulin, and albumin, that can be separated from the precipitates obtained from the

tively into the 2 liter flask. About 1 liter of water is added to the flask. The normality of an approximately 4 N solution of KOH is determined accurately. The number of cc. of this solution corresponding to 750 cc. of 4 N KOH is then added to the 2 liter flask. The solution is made up to approximate volume and warmed gently. When the phosphate has completely dissolved, the solution is cooled to room temperature and made up to volume, care being taken that the volume is correct after shaking. The resulting solution is almost always slightly murky. If so, it is filtered. Apparently because the solution is almost completely saturated filtration may cause a slight change in the phosphate concentration. But we have never by colorimetric methods found a measurable variation in the pH. To check the phosphate concentration of the solution the content is checked by the method of Fiske and Subbarow (27).

<sup>3</sup> 0.5 cc. of saturated sodium citrate per 100 cc. of whole blood. Immediately following collection, the cells were separated by centrifuging. In the experiments on horse plasma the plasma as soon as separated was added to the phosphate solutions. In the experiments on human plasma the plasmas were placed in the ice box overnight and added to the phosphate solution the following morning after being brought to 25°.

<sup>4</sup> Samples of filtrates from the precipitation flasks were allowed to remain at 25° for an added 48 hours without a further precipitation occurring.

different portions of the curves. In so doing we wish to avoid intimating that the protein complexes precipitated in our experiments are the purified proteins obtained by repeated fractionation. We, therefore, where there is any likelihood of ambiguity, have referred to the fractions of the curves as being identified with the fibrinogen, euglobulin, etc., complex or fraction.

The procedure involved in the experiments probably alters the protein complexes so that we are not dealing with the component systems existing in serum. As the procedure is milder and occupies a lesser time interval than that employed in the experiments of Svedberg or Sørensen, we may assume with reason that such changes in the component systems that have occurred are reversible and do not represent denaturation.

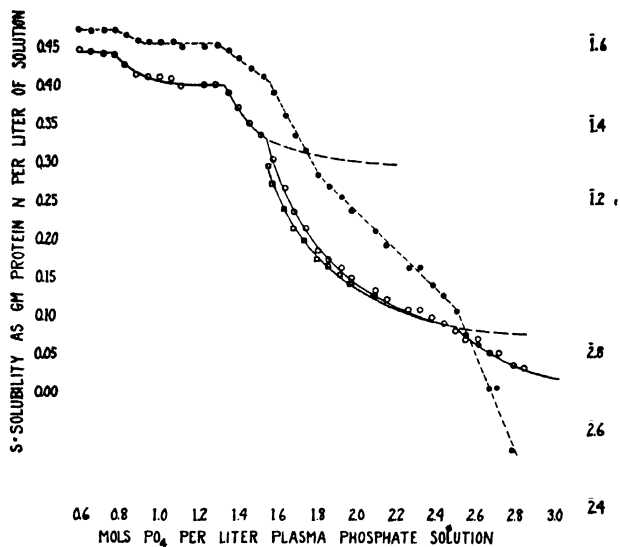
#### *Experiments on Horse Plasma*

Graphs I and II present the solubility curves of the plasma proteins from two different horses when portions of plasma were added to phosphate solutions of equimolecular amounts of dibasic and monobasic potassium phosphate. The procedure was carried out as given above in the outline of the experiments. The abscissa represents the molar concentrations of phosphate in the plasma phosphate solutions calculated from the dilutions made from the original 3 molar phosphate solution and the dilution resulting from the addition of the plasma to each particular phosphate solution. In two experiments the plasma phosphate solutions were analyzed for phosphate by the method of Fiske and Subbarow (27) and the results agreed within the expected experimental error with the concentrations as calculated. To simplify the experimental work we, therefore, eliminated the phosphate analyses except as checks on the 3 molar solutions and have throughout used the calculated concentrations in the graphs. The ordinate on the left represents the solubility of protein nitrogen expressed as gm. of nitrogen per liter of filtrate.<sup>5</sup> The ordinate on the right represents the logarithm of the solubility.

In Graphs I and II the curves through the outlined circles repre-

<sup>5</sup> In these experiments the concentrations of both phosphate and protein in the filtrates are expressed for convenience as mols per liter. From a theoretical standpoint it might be argued that protein solubility be expressed as mol fraction and salt concentration as ionic strength per liter, *i.e.*

sent the solubility curves. The broken line curves through the solid black dots represent the logarithms of the solubilities. It will be noted that there are breaks in this logarithmic curve not only at the points of break in the solubility curve, but also in the case of the pseudoglobulin portion at other points; for curves representing individual fractions are logarithmic only when the



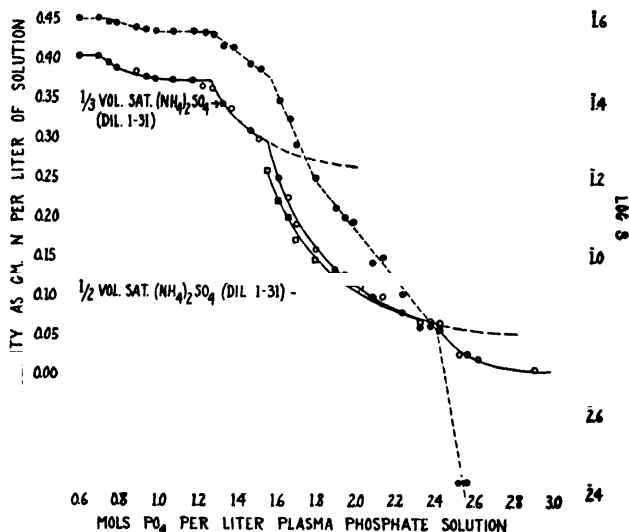
GRAPH I. Horse plasma, 20 ; dilution 1:31; Experiment 1.  $\circ$  = solubility curve;  $\square$  = pseudoglobulin curve corrected by subtracting euglobulin nitrogen;  $\bullet$  = logarithm of solubility.

solubility figures are so expressed as to result in such curves approaching zero asymptotically. In Graphs I and II the albumin fraction alone is so plotted. The euglobulin and pseudoglobulin portions of the logarithmic curve are such that, if the points are joined by straight lines and the curve is extensive enough, breaks result with the slope always becoming less. But any break in the logarithmic curve where the slope becomes greater must mean

Debye's  $\Gamma/2$ . Green (26) has shown that hemoglobin solubility expressed in such terms yields, within the experimental error, values of  $K$ , identical to those obtained when the molar terms here used are employed. In our systems the molar concentrations recorded are always one-half the ionic strength per liter, and hence should cause no confusion.

the precipitation of another protein fraction. This, we feel, has enabled us to detect with greater accuracy than otherwise possible the points of significant break in the solubility curves of the plasmas examined.

In the two experiments shown here and in three more on horse plasma the points of significant breaks occurred at phosphate



GRAPH II. Horse plasma, 25°; dilution 1:31; Experiment 2. ○ = solubility curve; □ = pseudoglobulin curve corrected by subtracting euglobulin nitrogen; ● = logarithm of solubility.

concentrations of between 1.20 to 1.35, 1.5 to 1.6, and 2.4 to 2.5 molar. Assuming that horse plasma contains protein complexes from which fibrinogen and the three serum proteins, euglobulin, pseudoglobulin,<sup>6</sup> and albumin, can be salted out, the data present curves very similar to a hypothetical solubility curve constructed

<sup>6</sup> Howe (19) working with calf sera reports evidence for two pseudoglobulins. We have determined the protein solubility on horse and human plasmas in 17.7 per cent  $\text{Na}_2\text{SO}_4$  and find no evidence of breaks in the phosphate curves at the corresponding protein solubility. Haslam (11) working with ox serum was unable to separate the pseudoglobulin into two proteins. Sørensen (17) divides the serum globulins of the horse into euglobulin and pseudoglobulin.

on the assumption that the individual protein complexes separated out similarly to pure proteins in concentrated salt solutions.

The first portion of the solubility curve in each graph corresponding to phosphate concentrations from 0.7 to 1.1 molar we believe represents the precipitation of the fibrinogen complex. Its identification as fibrinogen does not rest solely on its being the first fraction to precipitate out. Its salting out range agrees with that of fibrinogen in similar phosphate solutions reported by Florin (25). Furthermore, it represents in our experiments an average of 7 per cent of the total plasma proteins, which agrees with the reported values for fibrinogen in horse plasma of from 5 to 10 per cent. As no measurable precipitation occurs from 1.1 molar phosphate to 1.2 molar, an extension of the fibrinogen curve logarithmically indicates a negligible solubility of the fibrinogen complex in 1.2 molar phosphate solutions, and therefore a negligible amount of fibrinogen precipitated at higher phosphate concentrations.

Unfortunately there are no studies available for comparing the precipitation ranges of the serum protein complexes in phosphate solutions with the other breaks in the solubility curves. We, therefore, for the time being assume that the other breaks represent the precipitation of component systems from which euglobulin, pseudoglobulin, and albumin complexes, in the order corresponding to their known relative solubilities, can be separated.

From the data presented in Graphs I and II together with slight modifications to include the three experiments not presented graphically, the next protein fraction, the euglobulin complex, precipitates over the phosphate concentrations from 1.2 to 1.6 molar. The pseudoglobulin complex precipitates over the phosphate concentrations from 1.5 to 2.5 molar. And the albumin complex precipitates over the concentrations from 2.4 to 3.0 molar. From the analytical data the single phosphate concentrations which would approach with minimum error the points of break in the solubility curve of a given horse plasma are 1.1 molar, 1.5 molar, and 2.4 molar. But it is clear from the nature of the solubility curves that, with the exception of the concentration 1.1 molar, solubility determinations carried out at these concentrations do not represent the points of zero solubility of individual fractions.

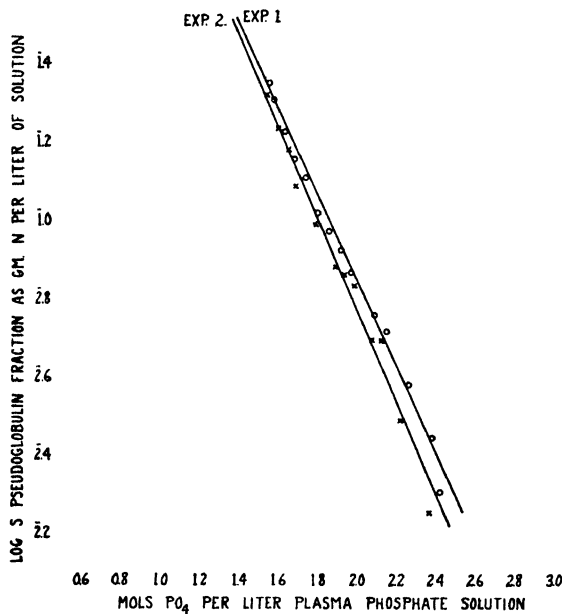
The broken line curves continuing the individual portions of the solubility curves corresponding to the precipitation of the euglobulin and pseudoglobulin fractions represent the extension of these fractional curves. Assuming that these extensions approximate the solubilities of the respective fractions, we see that the solubility of the euglobulin complex in the filtrate of 1.5 molar phosphate is considerable, roughly 25 per cent, and that the solubility of the pseudoglobulin complex in the filtrate of 2.4 molar phosphate, though less, is not negligible.

The analytical solubility curve over the pseudoglobulin range is not the true solubility curve of the pseudoglobulin complex because of the inclusion of euglobulin nitrogen in these determinations. The lower curve over the pseudoglobulin range in each graph represents the corrected pseudoglobulin complex solubility, the euglobulin complex nitrogen having been subtracted from the analytical data. It is, of course, this lower curve that theoretically is extendible logarithmically.

Graph III presents the logarithms of the corrected pseudoglobulin complex solubilities of the two experiments on horse plasma (Graphs I and II) plotted against the salt concentrations. In determining the pseudoglobulin solubilities a correction for the euglobulin complex precipitation over the pseudoglobulin range had to be introduced. In doing this we used the same logarithmic curve for the euglobulin fraction in each experiment. This coincided with the experimental data, and had to be done were a comparison of the pseudoglobulin curves to be made, as the limited length of the euglobulin curve and the distribution of the experimental points allowed considerable variation in drawing the curves of this fraction. The slope of the lines determines the  $K_s$  for each experiment. As  $K_s$  is slightly varied by altering the points of zero solubility for the pseudoglobulin complex, the significance of the data must be considered in terms of a first approximation. The closeness with which the data fall along the individual straight lines favors the validity of the logarithmic extensions as made in Graphs I and II and indicates that the selected points of zero solubility are not far from that which would result from a correct logarithmic extension and that the  $K_s$ 's are not grossly far from correct values.

Graph III shows that the slopes of the two lines or  $K_s$ 's agree

closely. We should not expect the solubilities to be the same, as Experiment 1 was carried out at 20° and Experiment 2 at 25°. Green (26) has shown that the solubility of several proteins varies with temperature because of the dependence of Cohn's intercept constant,  $\beta$ , on temperature.



GRAPH III. Logarithmic plot of pseudoglobulin of horse plasma from Experiments 1 and 2.

The albumin curve has not been corrected because of the small correction as compared to the analytical errors involved over the albumin portion of the solubility curve.

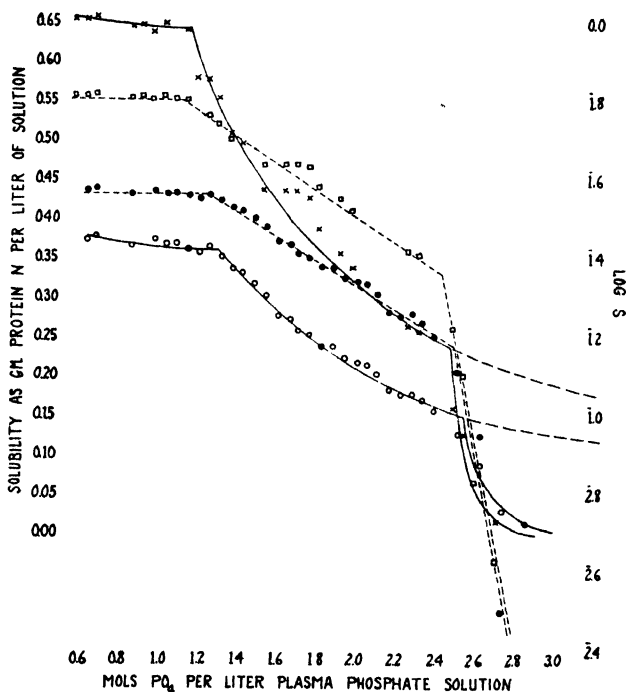
In Graph II we have indicated the protein solubilities determined on that plasma at one-third and one-half saturation by volume with ammonium sulfate<sup>7</sup> at a plasma dilution of 1:31.

<sup>7</sup> The protein nitrogen determinations were carried out by heat-coagulating 10 cc. of the ammonium sulfate plasma filtrate, by washing the coagulated protein precipitate with hot water until the washings contained no sulfate as measured by the precipitation of barium sulfate, and by determining the nitrogen of the protein precipitate by the macro-Kjeldahl method.

This is higher than the usual dilution of plasma when working with ammonium sulfate solutions, and will be commented on later.

### *Experiments on Human Plasma*

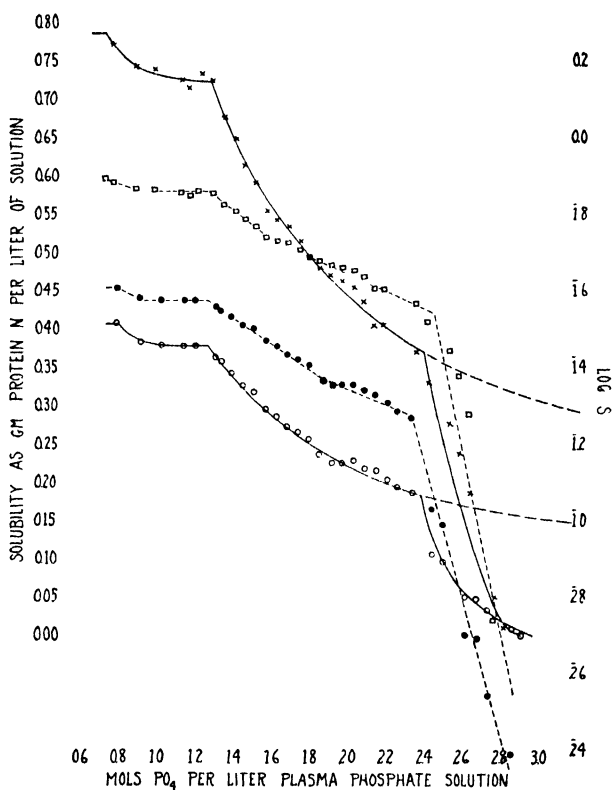
Graphs IV and V present solubility curves on human plasmas as determined in Experiments 6, 7, 8-A, and 8-B.



GRAPH IV. Human serum; Experiments 6 and 7.  $\circ$  = solubility;  $\bullet$  =  $\log S$ ; Experiment 6; dilution 1:31 (without chloroform).  $\times$  = solubility;  $\square$  =  $\log S$ ; Experiment 7; dilution 1:16 (with chloroform).

The plasmas of Experiments 6 and 7 clotted slightly so that in these experiments the fibrinogen fraction of the curve is not correct. Experiment 8-B, Graph V, gives the fibrinogen complex content of this human plasma as 8.2 per cent of the total plasma protein. Examination of the curves of Experiment 6, Graph IV, shows that no break can be detected at or near the phosphate concentration of 1.5 molar. The logarithmic plot also gives no con-

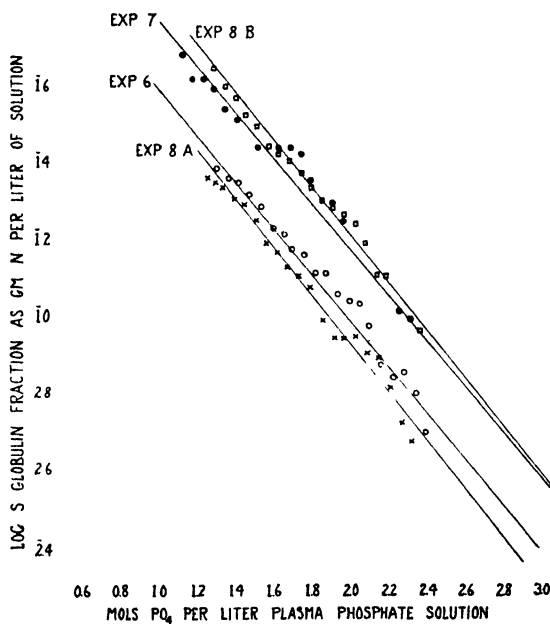
vincing indication of a break over the entire globulin range. This plasma also differs from the horse plasmas examined by its larger content of albumin complex. This increase in albumin complex with a lesser globulin complex results in a higher albumin to globulin ratio than in the horse plasmas.



GRAPH V. Human plasma; Experiment 8.  $\circ$  = solubility;  $\bullet$  =  $\log S$ ; Experiment 8-A; dilution 1:31.  $\times$  = solubility;  $\square$  =  $\log S$ ; Experiment 8-B; dilution 1:16.

The decreased globulin content we felt might have been responsible for the curve not showing any break corresponding to passing from the precipitation of euglobulin complex to pseudoglobulin complex. So in Experiment 7, we changed the experimental method by adding 1 cc. of plasma to 15 cc. of the phosphate

solution. This, of course, gave a greater range of protein concentrations in the solubility curve. But examination of the solubility curve or the logarithmic curve of Experiment 7 gives no convincing evidence of the precipitation of two protein fractions between 1.1 and 2.4 molar phosphate concentrations. The solubility curves of Experiments 8-A and 8-B, Graph V, confirm the absence of positive evidence of this type for two globulin fractions. Experiments 8-A and 8-B were performed simultaneously, with



GRAPH VI. Logarithmic plot of globulin of human plasma from Experiments 6, 7, 8-A, and 8-B.

plasma from the same patient. In Experiment 8-A 0.5 cc. of plasma was added to 15 cc. of phosphate solution; while in Experiment 8-B 1 cc. of plasma was added to 15 cc. of phosphate solution. In these two experiments, in which the plasma contained more albumin than previously encountered, the break in the solubility curve caused by what we assume is the precipitation of albumin complex comes at a phosphate concentration slightly less than 2.4 molar. This coincides with what might be expected

theoretically from Cohn's equation. The fact necessitates our moving the phosphate concentration that approaches the globulin to albumin point of break with minimum error to 2.3 molar when dealing with human plasma.

We purposely refrain from describing in this paper the albumin portion of the curves in detail. In the experiments on horse plasma the albumin complex concentrations are so low that the curves are too short to warrant analysis. In the experiments on human plasmas the solubility data of the albumin portion of the curves do not warrant the drawing of definite conclusions due to the divergence of many of the points in this portion of the curve of Experiment 8-B from a curve similar to that of the other experiments.

TABLE I

*Effect of Serum Dilution on Serum Protein Precipitation at  $\frac{1}{3}$  and  $\frac{1}{2}$  Volumes Per Cent Saturation by Volume with Ammonium Sulfate (Horse Serum)*

Dilution	Volumes per cent saturation	N per sample of filtrate	Solubility of protein N as gm. per liter solution	Soluble protein N per 100 cc. serum
		mg.		gm.
1:10	33.3	12.93	1.293	1.263
1:16	33.3	8.36	0.836	1.308
1:31	33.3	4.54	0.454	1.378
1:5	50.0	6.20	0.620	0.280
1:10	50.0	3.36	0.336	0.306
1:16	50.0	2.10	0.210	0.306
1:31	50.0	1.08	0.108	0.305

Graph VI presents the solubilities of the globulin fractions of the four experiments on human plasma plotted as described for Graph III.

It is seen that the agreement as regards slope is close. Though the closeness with which the experimental points follow the straight lines is not as good as in Graph III, it is sufficient to warrant the construction of the lines as drawn.

Table I shows the effect of plasma concentration on the protein solubilities of solutions of one-third and one-half volumes saturation with ammonium sulfate. The determinations were carried out on a single specimen of horse serum. In the fourth column is shown the change in protein nitrogen solubility in the plasma salt

solutions. In the last column is shown the effect on the protein solubility calculated as gm. of soluble protein nitrogen per 100 cc. of serum.

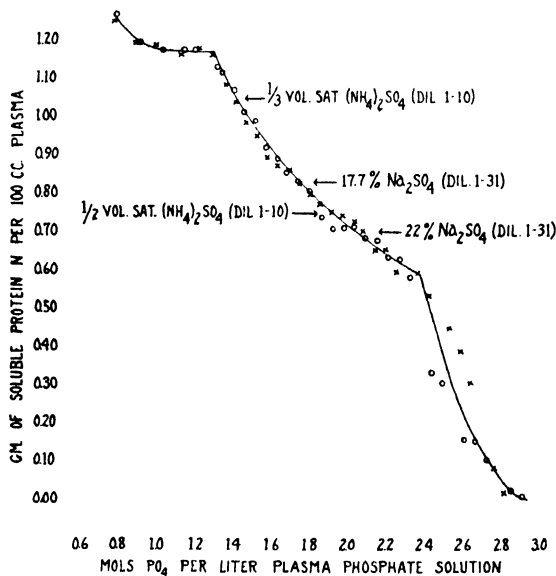
#### DISCUSSION

In Graph II the solubilities at one-third and one-half saturation with ammonium sulfate are designated as a means of identifying points on the phosphate curve with the commonly used concentrations of this standard salting out salt. Ordinarily, however, precipitation with ammonium sulfate is carried out with a plasma dilution of not more than 1:10. We, therefore, examined the effect of dilution of the plasma on the solubilities at these ammonium sulfate concentrations. The data of Table I show that at one-third saturation a change from a dilution of 1:10 to one of 1:31 causes an increase in the soluble protein per 100 cc. of serum. The solubility at one-third saturation with ammonium sulfate on Graph II cannot, therefore, be considered as the solubility at a standard ammonium sulfate concentration. At one-half saturation, however, changes from a plasma dilution of 1:10 up to one of 1:31 did not alter the soluble protein per 100 cc. of serum. Hence the solubility at this saturation with ammonium sulfate may be considered as a standard of reference. That the solubility at one-half saturation with ammonium sulfate is greater than that at the globulin to albumin point of break on the phosphate curve (Graph II) does not necessarily mean that the fractionation in the two solutions is different. For Ruszczynski (22) presented evidence indicating that 60 per cent saturation with ammonium sulfate corresponds to the beginning of albumin precipitation in horse serum ammonium sulfate solubility curves.

In Graph V the curves of Experiments 8-A and 8-B show the effect of the change in plasma concentration on protein solubility. Sørensen (17) observed a similar dependence of the globulin solubility on total globulin employed in his studies on the solubility of horse globulins. Because of this effect one cannot hope by increasing the plasma concentration to increase significantly the accuracy of estimating the concentration of an individual plasma protein complex by reducing the *relative* amount of that protein complex left in solution at the salt concentration selected for precipitation. This is presented quantitatively very clearly by plotting

the data of Experiments 8-A and 8-B so that the abscissa represents gm. per cent of soluble protein nitrogen in the plasma itself, not plasma-phosphate solutions. The data so plotted are presented in Graph VII.

Since the resulting curves of the two experiments coincide within the probable limit of experimental error, no advantage has resulted from the analysis at the higher plasma concentration.



GRAPH VII. Human plasma; Experiment 8. O = from precipitations at 1:31 dilutions. X = from precipitations at 1:16 dilutions.

In Experiments 8-A and 8-B we wished to detect breaks in the globulin curve at points generally accepted as being near the beginning precipitation of pseudoglobulin and albumin and we therefore used a 1:10 dilution of the plasma for the ammonium sulfate precipitations. The soluble protein nitrogen per 100 cc. of serum at one-third and one-half saturation by volume with ammonium sulfate and in 17.7 and 22 per cent sodium sulfate solutions (these latter being the solutions used by Howe (19) as coinciding respectively with the beginning precipitation of his Pseudoglobulin II and albumin) is designated on Graph VII. The data show no convincing evidence of a significant break at

the solubilities corresponding to that found in any of these solutions.

Green (26) in describing the solubility behavior of egg albumin and carboxyhemoglobin in concentrated solutions of strong electrolytes showed that the  $\beta$  term of Cohn's equation depends upon temperature, pH, and particular protein, whereas the  $K_s$  term varies with the electrolyte and protein. In the solutions of purified proteins, such as are considered in Green's paper, the solubility is independent of the amount of protein used in the experiment. In our experiments the solubility of the plasma protein complexes is dependent on the concentration of plasma. As already mentioned, Sørensen, in experiments on serum albumin, in which the solubility varied with the amount of albumin used in the experiments, found a linear relation between the logarithm of the solubility and the salt concentration. The data of Experiments 8-A and 8-B, as plotted in Graph VI, suggest that the plasma concentration affects the  $\beta$  rather than the  $K_s$  term.

In so far as Cohn's  $K_s$  can be considered as a characterization of a plasma protein fraction, Graph VI presents evidence that but one protein complex is being precipitated over the globulin range. And in so far as the slopes of the straight lines of Graph VI are different from the slopes of the lines of Graph III, the data suggest that the globulin of human plasma is different from the pseudoglobulin of horse plasma. The need for calling attention to such differences in the solubility behavior of the globulin fractions from sera of the two species is shown by the frequency with which values for euglobulin and pseudoglobulin in human serum determined by methods based on calf or other sera analyses have been reported in medical literature.

Since our experiments give no evidence that the linear relation between the logarithm of the solubility of an individual protein fraction and salt concentration does not apply to the precipitation of the protein complexes as separated in fractional precipitation, and since these experiments and those of Sørensen present evidence favoring the applicability of the relation, we have calculated the concentrations of the protein fractions in plasma by logarithmic extension of each fraction's curve to zero solubility for that fraction.

Table II shows the comparison of the estimated concentrations

TABLE II

*Comparison of Serum Protein Concentrations As Determined by Precipitation with 1.5 and 2.4\* M  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  of pH 6.5 and by Logarithmic Extension of Phosphate Solubility Curves and by Precipitation with  $\frac{1}{3}$  and  $\frac{1}{2}$  Volumes Per Cent Saturation with  $(\text{NH}_4)_2\text{SO}_4$  at 25° and with 14.5 and 22 Per Cent  $\text{Na}_2\text{SO}_4$  at 37°*

The results, except for the albumin-globulin ratio, are expressed as gm. per cent protein derived by multiplying the protein nitrogen by 6.25.

Experiment No.	Protein fraction	$\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$		$(\text{NH}_4)_2\text{SO}_4$ precipitation	$\text{Na}_2\text{SO}_4$ precipitation
		At 1.5 and 2.4 M	By loga- rithmic exten- sion		
1. Horse, diphtheria	Euglobulin	1.28	2.04		
	Pseudoglobulin	4.68	4.36		
	Albumin	1.80	1.36		
	Albumin-globulin ratio	0.30	0.21		
2. Horse, scarlet fever	Euglobulin	1.43	2.23	0.57†	
	Pseudoglobulin	4.56	4.07	4.66†	
	Albumin	1.28	0.97	2.03†	
	Albumin-globulin ratio	0.21	0.15	0.39	
3. Horse, normal	Euglobulin	0.94	1.38		
	Pseudoglobulin	3.88	3.88		
	Albumin	2.18	1.74		
	Albumin-globulin ratio	0.45	0.33		
4. Horse, normal	Euglobulin	1.36	1.74	0.11†	0.11
	Pseudoglobulin	2.61	2.81	2.57†	2.74
	Albumin	2.03	1.45	3.32†	3.15
	Albumin-globulin ratio	0.51	0.32	1.24	1.10
6. Human, cardiac failure	Globulin	4.09	5.06	3.21‡	3.14
	Albumin	3.10	2.13	3.98‡	4.05
	Albumin-globulin ratio	0.76	0.42	1.24	1.10
7. Human, cardiac failure	Globulin	3.99	4.84	2.69§	
	Albumin	2.45	1.60	3.75	
	Albumin-globulin ratio	0.61	0.33	1.39	
8. Human, hyperten- sion	Globulin	3.74	4.65	2.67†	3.00
	Albumin	3.62	2.71	4.69†	4.36
	Albumin-globulin ratio	0.97	0.58	1.76	1.45

\* For the experiments on human serum 2.3 M was taken instead of 2.4 M.

† Dilution of plasma 1:31.

‡ Dilution of plasma 1:10.

§ Dilution of plasma 1:3.

|| Dilution of plasma 1:4.

of the protein complexes of several horse and human plasmas according to whether the concentrations of the individual protein fractions were determined by precipitation with 1.5 and 2.4<sup>a</sup> molar  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  of pH 6.5 or by logarithmic extension of the phosphate solubility curves or by precipitation with one-third and one-half saturation by volume with ammonium sulfate or with 14.5 and 22 per cent sodium sulfate according to the method of Howe (19).

By the method of logarithmic extension of the phosphate curves Experiments 3 and 4 give an albumin to globulin ratio for the normal horse of 0.33. In Experiment 4 the ratio by Howe's method is 1.1. Van Slyke, Hastings, Hiller, and Sendroy (28), using Howe's method, give a ratio of 0.8 for a normal horse. Analysis of Ruzsyczynski's curves in ammonium sulfate solutions by logarithmic extensions indicates a ratio of approximately 0.43. Mellanby (29), using alcohol-water mixtures, concluded that in horse serum about 3 per cent of the total protein was euglobulin, 85 per cent what he called albumin A and 12 per cent albumin B. If we assume the first two fractions represent globulin and the last fraction albumin, the ratio from his method of analysis is 0.14. Sørensen (18), in determining the albumin and globulin content of horse serum protein powder by repeated fractionation, obtained an albumin to globulin ratio of 0.37.

It is interesting to note the low albumin to globulin ratios in the plasmas of the horses producing diphtheria and scarlet fever antitoxin.

#### SUMMARY

In this paper we have outlined the evolution of our knowledge concerning the separation and identification of the plasma proteins, and have pointed out advantages of using phosphate solutions in experiments concerning their solubility behavior.

Solubility curves of the plasma proteins of horse and human plasmas in concentrated phosphate solutions of pH 6.5 and temperature of 25° are presented.

The effect of plasma concentration on the protein solubility in phosphate and ammonium sulfate solutions is demonstrated and discussed.

<sup>a</sup> For the experiments on human serum 2.3 M was taken instead of 2.4 M.

By assuming for each portion of the protein solubility curve of plasma a linear relation between the logarithm of protein solubility and the salt concentration, an attempt has been made to determine the solubilities of the less soluble plasma protein fractions at the concentrations selected for fractional precipitation and to estimate the concentrations of the individual plasma protein fractions.

A comparison is made of the individual protein content of several horse and human plasmas according to whether the concentrations of the individual protein fractions were estimated by precipitation with selected phosphate solutions or by logarithmic extension of the phosphate solubility curves or by precipitation with the commonly used ammonium sulfate and sodium sulfate solutions.

The difference between the precipitation curves of horse and human plasmas is demonstrated.

We wish to acknowledge our indebtedness to Dr. E. J. Cohn, who in conversation with one of us called attention to the desirability of such a study as presented here, and who advised us throughout the early part of the work.

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## PHOSPHATASE STUDIES

### I. DETERMINATION OF INORGANIC PHOSPHATE. BEER'S LAW AND INTERFERING SUBSTANCES IN THE KUTTNER-LICHTENSTEIN METHOD\*

By AARON BODANSKY

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We have long used the Benedict-Theis method for the determination of serum "inorganic phosphate," and have found it particularly satisfactory because of the slight deviation from Beer's law. However, the use of heat rendered this method unsuitable for the determination of inorganic phosphate in the presence of glycerophosphate, as in the serum phosphatase determination. We found the Kuttner-Cohen method (1), as modified by Kuttner and Lichtenstein (2) and Raymond and Levene (3), highly satisfactory and capable of giving, *in the absence of interfering substances*, results accurate to within 1 or 2 per cent, depending upon the analyst, *after proper correction for the very considerable deviation from Beer's law* (4). Errors, however, are caused by the presence of trichloroacetic acid, as in the determination of inorganic phosphate in serum, and by glycerophosphate, as in the phosphatase determination. With corrections for these errors, the great accuracy of which the Kuttner-Lichtenstein method is capable has been maintained. This has been particularly useful in the determination of phosphatase, which is a determination by difference, and in which the omission of the corrections would lead to significant errors.

\* The method with the corrections described in this paper was demonstrated at the Twenty-sixth annual meeting of the American Society of Biological Chemists at Philadelphia, April, 1932, and briefly outlined in the presentation of a study based upon it (Bodansky, A., Jaffe, H. L., and Chandler, J. P., *J. Biol. Chem.*, **97**, p. lxvi (1932)).

Our procedure is identical with Kuttner and Lichtenstein's, except for the stated changes in reagents and procedure.

### *Method*

*Stock Solutions. Sulfuric Acid, 10 N*—Keep in the refrigerator until it is to be used.

*Sodium Molybdate Solution, 7.5 Per Cent*—Molybdic acid (Eimer and Amend, T. P., Special, "ammonia- and phosphate-free"), 90 gm.; 5 N solution of sodium hydroxide, 250 cc. Dissolve in a 2 liter volumetric flask and dilute to volume; mix. The solution should be *faintly* alkaline to phenolphthalein. Let stand and decant for use. This solution is preferred to Kuttner and Lichtenstein's, which is prepared by dissolving sodium molybdate, because the latter has frequently given appreciably blue blanks.

*Stannous Chloride Solution, 60 Per Cent*—Dissolve 15 gm. of stannous chloride (Eimer and Amend, T. P.) in concentrated hydrochloric acid, making up to the mark in a 25 cc. volumetric flask. Let stand in the refrigerator. This solution is made up monthly. We found that more consistent results over a wider range of inorganic phosphate values could be obtained with this more concentrated reagent, in the use of which we followed Raymond and Levene (3).

*Standard Phosphate Stock Solution*—Dissolve 110 mg. of potassium acid phosphate (LaMotte, buffer grade) in water, add 1 cc. of concentrated sulfuric acid, and dilute to 250 cc. 10 cc. contain 1 mg. of P.

*Solutions for Use in Analysis.*<sup>1</sup> *Molybdate Reagent*—Cold 10 N sulfuric acid, 1 volume; 7.5 per cent molybdate stock solution, 1 volume, added quickly, while mixing; water, 2 volumes. (By

<sup>1</sup> We have used this method in the determination of "inorganic phosphate" of plasma or serum, and in the serum phosphatase determination. The filtrate for the determination of serum inorganic phosphate is prepared as follows: serum, 1 cc.; 5 per cent trichloroacetic acid, 9 cc.; mix, let stand a few minutes, and filter through No. 44 Whatman paper. A lower dilution may be used, if preferred, when inorganic P is expected to be less than 3 mg. per 100 cc. The preparation of filtrates for phosphatase determinations involves many considerations which cannot be treated here. They were previously stated briefly (Bodansky, A., *Proc. Soc. Exp. Biol. and Med.*, **28**, 760 (1931)). A full statement and discussion of the principles involved and the details followed is to be published.

omitting the water, a more concentrated reagent is obtained for use with 7 cc. aliquots.) Make up daily. When the above precautions are not observed, the reagent may have a yellow tinge, resulting in an appreciably blue blank after the addition of stannous chloride.

*Dilute Stannous Chloride Reagent*—Dilute the 60 per cent stock solution 200 times. Make up daily, and keep in a glass-stoppered bottle in the refrigerator between analyses.

*Standard Phosphate Solution*—Phosphate stock solution, 10 cc.; dilute to 250 cc. and preserve with a drop of toluene. 5 cc. contain 0.02 mg. of P.

*Reagent Blanks*—Trichloroacetic acid, 5 cc.; molybdate reagent, 4 cc.; mix; add dilute stannous chloride reagent, 1 cc.; mix. The resulting mixture is colorless, or at most faintly blue or green. The blank is not only a check upon the quality of the chemicals used but also upon the proper preparation of the day's molybdate reagent.

*Analysis*<sup>1</sup>—A single series for a colorimetric comparison may conveniently contain twenty test-tubes (18 or 20 mm. by 150 mm.), including two or three containing *one* standard (we prefer 0.02 mg. of P). The standards and the unknown aliquots (containing between 0.012 and 0.036 mg. of P) are made up with water to 5 cc., if necessary; 4 cc. of the molybdate reagent are added to each tube.<sup>2</sup> The contents of each are mixed by tapping, then 1 cc. of the stannous chloride reagent is added, the contents being immediately mixed by a single inversion. The two or three standards are checked against each other, as a matter of routine, both at the beginning and the end of each series of comparisons. Duplicate readings are recorded in the usual manner.

Analyses are performed in duplicate.

*Calculations*—The usual formulæ for calculation do not include a correction for the deviation from Beer's law. This deviation, which amounts to 20 per cent of the difference between the P content of the unknown and of the standard, may be corrected for by the use of the formula

$$\frac{0.48}{\text{Reading of unknown}} - 0.0040 = \text{mg. P in aliquot}$$

<sup>1</sup> When 7 cc. aliquots are used (with 2 cc. of the concentrated molybdate reagent) it is advisable to dilute the standard tubes to 7 cc.

For convenience, however, the values corresponding to any given reading have been calculated and tabulated (see Table I). In

TABLE I

*Inorganic P in Aliquot, at Stated Colorimetric Readings, Corrected for Deviation from Beer's Law. 0.02 Mg. Standard Set at 20 Mm.*

Mm....	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
mm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
8	0.0560	0.0552	0.0545	0.0538	0.0531	0.0525	0.0518	0.0512	0.0505	0.0499
9	493	487	482	476	471	465	460	455	450	445
10	440	435	431	426	422	417	413	408	404	400
11	396	392	389	385	381	377	373	370	367	363
12	360	357	353	350	347	344	341	338	335	332
13	329	326	324	321	318	316	313	310	308	305
14	303	301	298	296	293	291	289	287	284	282
15	280	278	276	274	272	270	268	266	264	262
16	260	258	256	254	253	251	249	248	246	244
17	242	241	239	237	236	234	233	231	230	228
18	227	225	224	222	221	220	218	217	215	214
19	213	211	210	209	207	206	205	204	203	201
20	200	199	198	197	196	194	193	192	191	190
21	189	188	186	185	184	183	182	181	180	179
22	178	177	176	175	174	173	172	171	170	170
23	169	168	167	166	165	164	163	163	162	161
24	160	159	158	158	157	156	155	154	153	152
25	152	151	151	150	149	148	147	147	146	145
26	145	144	143	143	142	141	140	140	139	138
27	138	137	137	136	135	135	134	134	133	132
28	132	131	130	130	129	128	128	127	127	126
29	126	125	124	124	123	123	122	122	121	121
30	120	119	119	118	118	117	117	116	116	115
31	115	114	114	113	113	112	112	111	111	110
32	110	110	109	109	108	108	107	107	106	106
33	105	105	105	104	104	103	103	102	102	102
34	101	101	100	100	100	099	099	098	098	098
35	097	097	096	096	096	095	095	095	094	094
36	093	093	093	092	092	092	091	091	090	090

serum analyses the aliquot value may be converted into mg. of inorganic P per 100 cc. of serum by the use of the formula

$$T \times \frac{V}{V'} \times 100 = \text{mg. P per 100 cc.}$$

where  $T$  equals the Table I value of the aliquot, corresponding to the colorimeter reading,  $D$  equals the dilution of the serum in the filtrate (usually 10 in serum phosphate determinations and 20 in the phosphatase determinations), and  $V$  equals the volume of the aliquot, in cc.

Table II shows the application of these corrections in a series of analyses over the range recommended. Near the limits of the

TABLE II  
*Corrections for Deviation from Beer's Law*

Known P	Uncorrected (C)*		Corrected (T)*	
	Phosphorus	Errors	Phosphorus	Errors
<i>mg</i>	<i>mg</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
0 0330	0 0305	-8 1	0 0326	-1 3
275	263	-4 5	276	0 4
220†	220	0	224	1.8
206†	206	0	207	0 5
185†	186	0 5	183	-1.1
165	172	4 0	166	0 6
144	152	5 3	142	-1 4
124	137	9 4	124	0

\* Solutions of potassium acid phosphate of known concentration were used.  $C$ , calculated from the readings of the unknown without corrections for deviation from Beer's law;  $T$ , Table I values, including corrections for the deviation from Beer's law. The errors were calculated from the ratio of *known P* to *value found*.

† If many similar series are analyzed the corrected values even within this range (90 to 110 per cent of standard) would show, in most cases, a smaller error than the uncorrected values, although the deviation within this range is small and in any one series the relation may be reversed, as above, by the error in colorimetric comparison.

range indicated in Table I greater errors may be expected (3 to 5 per cent).

*Trichloroacetic Acid and Glycerophosphate Corrections*—When trichloroacetic acid is present alone or with glycerophosphate, as in the determinations of serum inorganic phosphate and serum phosphatase, respectively, further corrections are necessary. The concentration of these substances has been so adjusted in these analyses as to permit the use of a convenient correction

factor. This factor is +1 per cent per cc. of serum P filtrate used in the analyses, and +2 per cent per cc. of phosphatase filtrate (maximum correction 10 per cent). (For greater precision, the following corrections may be used for trichloroacetic acid *plus* glycerophosphate: for 1 cc. of phosphatase filtrate or less, 3 per

TABLE III  
*Corrections for Trichloroacetic Acid and Glycerophosphate*

Known P	Volume	Trichloroacetic acid only				Trichloroacetic acid and glycerophosphate*			
		Uncorrected		Corrected		Uncorrected		Corrected	
		Value T	Error	Inor- ganic P	Error	Value T	Error	Inor- ganic P	Error
mg.	cc.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
0.0330	6					0.0298	-11	0.0328	0.6
247		0.0234	-6	0.0248	0.4	226	-9	249	0.8
206		192	-7	204	1.0	187	-10	206	0
165		152	-9	161	2.4	148	-12	163	1.2
124		114	-9	121	2.5	110	-13	121	2.5
0.0330	4	312	-6	324	1.9	300	-10	324	1.9
247		237	-4	246	0.4	229	-8	247	0
220		212	-4	220	0	204	-8	220	0
165		163	-1	170	3.0	151	-9	163	1.2
137		134	-2	139	1.5	127	-8	137	0
0.0247	2	240	-3	245	0.8	235	-5	247	0
206		201	-2	205	0.5	194	-6	204	1.0
165		161	-2	164	0.6	154	-7	162	1.9
124		119	-4	121	2.5	115	-8	121	2.5

Solutions of potassium acid phosphate of known concentration were used, containing in addition trichloroacetic acid, and trichloroacetic acid *plus* glycerophosphate, in concentrations equal to those used in serum inorganic phosphate and serum phosphatase determinations, respectively. Aliquots of 7, 5, 3, and 1 cc. yielded similar results, which are omitted here to save space.

\* See foot-note 3.

cent per cc.; for 2 cc. of filtrate, a 5 per cent correction; for 3 and 4 cc. of filtrate, 6 and 8 per cent, respectively; for 5, 6, and 7 cc. of filtrate, 10 per cent.<sup>3</sup>)

<sup>3</sup> Greater relative effects (and, of course, smaller absolute effects) are found when the smaller aliquots of glycerophosphate solution are used. All factors were determined in a large number of determinations for each

TABLE IV  
*Corrections for Trichloroacetic Acid and Glycerophosphate in Serum Analyses\**

Filtrate	Known inorganic P				Calculated inorganic P			
	Serum filtrate		Added P	Total P	Uncorrected		Corrected	
	Volume	Phosphorus			Value T	Error	Inorganic P	Error
	cc.	mg.	mg.	mg.	mg.	per cent	mg.	per cent
A	5						0.0210	
	4						168	
	4	0.0168	0.0063	<b>0.0231</b>	0.0224	-4.2	233	0.8
	3	126	095	<b>221</b>	217	-2.0	224	1.2
B	7						229	
	6						199	
	6	197	032	<b>229</b>	206	-11.2	227	-0.8
	5	165	063	<b>228</b>	208	-9.6	229	0.4
	3	099	126	<b>225</b>	212	-6.1	225	0
	2	066	158	<b>224</b>	214	-4.7	223	-0.5
C	5						235	
	5						238	
	5	237	066	<b>303</b>	273	-11.0	300	-1.0
	4	189	099	<b>288</b>	263	-9.5	284	-1.4
	3	142	132	<b>274</b>	253	-8.2	268	-2.3
	2	094	165	<b>259</b>	245	-5.7	257	-0.8
	1	047	196	<b>245</b>	237	-3.4	244	-0.4

The correction for the deviation from Beer's law is included in the "uncorrected" values *T* (taken from Table I). Filtrate A, prepared for a serum phosphate determination, contained trichloroacetic acid; Filtrates B and C, prepared for a phosphatase determination, contained glycerophosphate as well.

\* See foot-note 3.

These corrections may be applied to the final value (inorganic P per 100 cc.) or, as we have done in this paper, to the value *T*.

concentration of phosphate and for each aliquot volume (and, therefore, for a varying quantity of trichloroacetic acid or trichloroacetic acid *plus* glycerophosphate). The errors were calculated from the ratio of *known contents to value found* (yielding the percentage correction to be applied to the latter). The data serving as the basis for the corrections are too numerous to be reported. Typical series of analyses are shown in Tables III and IV. Duplicate determinations would yield averaged values for each sample, generally with smaller errors than shown in the tables.

Some values, before and after correction, are given in Tables III and IV.

#### DISCUSSION

The deviation from Beer's law is considerable even within the range for which Kuttner claims substantial accuracy—70 to 130 per cent of the concentration of the standard (5)—as may be seen from the inspection of Table I and of those values in Table II which fall within this range. The application of the corrections not only reduces the errors within this range, but extends considerably the range within which values accurate within 1 or 2 per cent may be obtained (60 to 180 per cent of the concentration of the standard). Even at the limits of the range indicated by Table I the results are accurate within 3 to 5 per cent.

The corrections for trichloroacetic acid and for glycerophosphate are essential. Within a certain narrow range (about 0.014 to 0.017 mg. of P) the errors sometimes balance; a limited number of determinations might lead to undue reliance upon uncorrected results.

The effect of trichloroacetic acid upon color intensity has been noted by Kuttner and Cohen, but greatly minimized (1); it has also been observed by G. T. Ori,<sup>4</sup> who balances this effect by adding trichloroacetic acid to the standards. A carefully established correction is, however, more generally useful, because it can be employed when aliquots of varying volume (and therefore mixtures of varying trichloroacetic acid content) are used in one series of analyses.

Table III illustrates also the effect of varying quantities of glycerophosphate (*plus* trichloroacetic acid) when aliquots of 1 to 7 cc. are employed. Errors up to 11 per cent after correction for deviation from Beer's law (and up to 17 per cent before this correction) are reduced to less than 2 per cent after the corrections are applied.

In determinations of inorganic phosphate liberated by the action of phosphatase—a value calculated from the difference between total inorganic P (in a filtrate from the incubated specimen containing glycerophosphate) and serum inorganic P (only trichloroacetic acid present)—the errors of the uncorrected values may be

<sup>4</sup> Personal communication.

increased further, as in all determinations by difference. The employment of a zero specimen containing glycerophosphate (6) does not avoid the error; on the contrary, the analytical error in the case of the larger aliquot of the zero filtrate necessarily used for analysis would be as great as — 10 per cent, due to its higher trichloroacetic acid and glycerophosphate content. This error could of course be corrected by the use of our factors. However, the employment of our corrections renders the zero specimen superfluous. We use instead a determination of serum inorganic phosphate, which is frequently of independent interest in clinical or experimental material.

The use of two or three tubes of one concentration of standard phosphate solution (0.02 mg. of P) rather than of one tube of each of several standards provides an adequate check on the standard and is more convenient. By the use of Table I satisfactory results are obtained with that standard when the unknowns range between 0.0120 and 0.0360 mg. of P.<sup>5</sup> It is common experience that the most accurate colorimetric comparisons are made within a certain range of color intensities. Therefore, when some results fall outside of the range which we have found satisfactory, we prefer to repeat the analyses with aliquots chosen to contain a quantity of P well within the range, rather than make comparisons with less or more concentrated standards.

#### SUMMARY

1. The deviation from Beer's law in the Kuttner-Lichtenstein method for determination of inorganic phosphate is considerable—20 per cent of the difference between the concentration of the unknown and the standard. A table of corrected values corresponding to the colorimetric readings obtained under standardized conditions may be conveniently substituted for the usual or for our modified formula.

2. When trichloroacetic acid or trichloroacetic acid *plus* glycerophosphate is present in the solution to be analyzed, large errors are caused by their effect on the intensity of the color developed. The conditions of the test have been standardized to make possible the use of convenient correction factors.

<sup>5</sup> We check each series by the insertion of two or more tubes of stock solutions of potassium acid phosphate covering a similar range, with results similar to those shown in Table I.

3. The Kuttner-Lichtenstein method for determination of inorganic phosphate, with slight modifications, and after corrections for the deviation from Beer's law and for the effects of trichloroacetic acid and glycerophosphate, yields highly accurate results in the presence of these interfering substances, as in the determination of serum inorganic phosphate and of serum phosphatase.

4. Inorganic phosphorus values ranging, within one series, between 0.0120 and 0.0360 mg. may be determined with an accuracy of 1 to 2 per cent, with the employment of one standard, 0.02 mg. of P; the determination of values outside of this range involves an error of 3 to 5 per cent.

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## TWO NEW COLOR TESTS FOR HEXOSES

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Color reactions of urea are so rare that there is particular interest in the method devised by Nakashima and Maruaka<sup>1</sup> for the determination of urea in blood. This method is based upon the production of a blue-violet color on addition of 0.1 cc. of urea solution to a mixture of 0.075 cc. of freshly distilled furfuraldehyde and 4 cc. of a solution of stannous chloride in 30 per cent hydrochloric acid. A study of the function of urea in this test shows that it induces rapidly, and at a low temperature, a reaction which can occur at the boiling point in its absence. This conclusion is based upon the following facts: (1) Furfuraldehyde gives a red color on boiling with 30 per cent hydrochloric acid. In the presence of urea the same color appears below the boiling point. (2) Furfuraldehyde, on warming with a solution of stannous chloride in hydrochloric acid, gives a red-violet to blue-violet color. The degree of blueness increases with increase in the concentration of the stannous chloride. The same color change occurs rapidly at room temperature in the presence of urea. (3) On standing at room temperature a mixture of furfuraldehyde with the solution of stannous chloride in hydrochloric acid slowly turns blue-black and deposits a black solid. This change also is much more rapid in presence of urea.

In the test of Nakashima and Maruaka the intensity of the final color is proportional to the concentration of urea. The function of urea, therefore, does not completely accord with that of a catalyst.

<sup>1</sup> Nakashima, Y., and Maruaka, K., *Deutsch. Arch. klin. Med.*, **143**, 318 (1924).

This test for urea can be used also for guanidine, with similar results.

Since the simple carbohydrates give derivatives of furfuraldehyde when heated with strong mineral acids, it was thought possible that a urea-stannous chloride-acid reagent might be used to produce color tests for sugars. Such a reagent has been made and is termed here the reagent containing urea.

*Reagent Containing Urea*—40 gm. of urea are dissolved in 80 cc. of 40 per cent (by volume) sulfuric acid. 2 gm. of stannous chloride are added and the mixture boiled vigorously until clear. After cooling, the volume is made up to 100 cc. with 40 per cent sulfuric acid. This reagent is stable for at least 1 year when kept at room temperature in a glass-stoppered bottle.

In using this reagent, 0.5 cc. of the sugar solution to be tested is mixed with 3 cc. of the reagent and the mixture heated to boiling and kept briskly boiling for about 45 seconds. It is then shaken vigorously. If the sugar tested be a ketohexose, a green-blue color appears and increases in intensity as the mixture cools. According to the concentration of the sugar the final color will be blue to blue-violet. If the sugar used be an aldohexose, a yellow or olive-green color appears on boiling for 45 seconds. Alternate boiling and shaking for 2 to 3 minutes produce a color distinctly redder than that given by a ketose and best described as amethyst.

Furfuraldehyde and the pentoses, arabinose and xylose, give only an intense yellow color with this reagent. The disaccharides give the colors of the simple sugars of which they are composed; thus maltose and lactose give the amethyst color of glucose, inulin gives the blue of levulose, and sucrose gives a mixed color. These more complex sugars react less easily than do the hexoses. The solution of urea and stannous chloride in 40 per cent sulfuric acid gives merely a pale orange color on prolonged boiling.

The lower limits of sugar concentration at which these colors appear are as follows: For levulose, 0.02 mg. in 3.5 cc. of test mixture gives a barely perceptible green-blue color; 0.04 mg. gives a distinct color; 0.08 mg. gives a fairly intense blue. For glucose, mannose, galactose, 0.5 mg. in 3.5 cc. of test mixture gives a green color, changing on further boiling into a perceptible amethyst.

The absorption spectra of the colors given with the reagent containing urea are shown in Fig. 1.

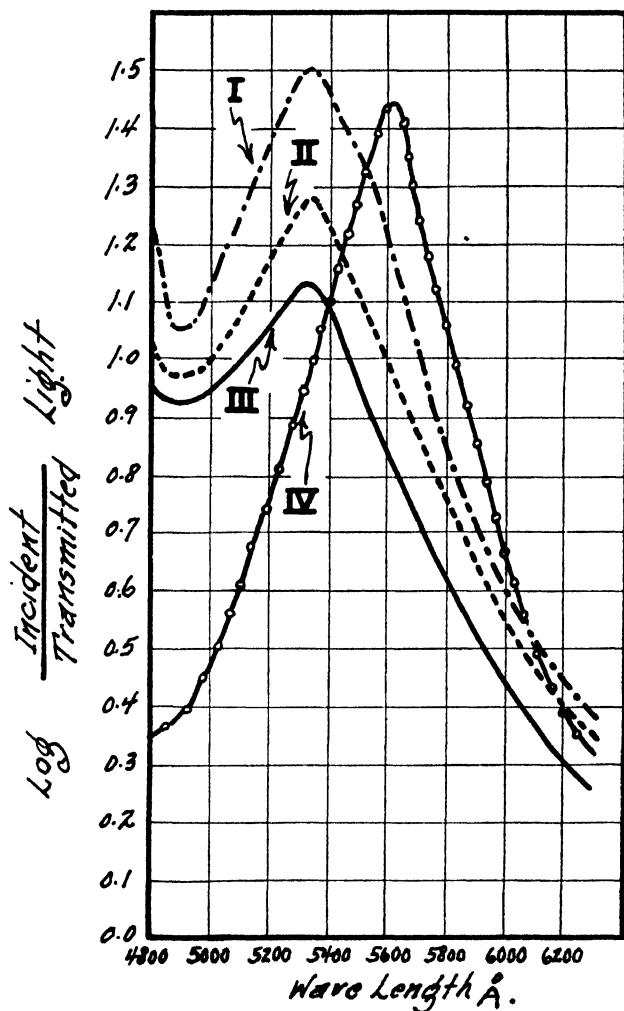


FIG. 1. Absorption spectra of color tests given with the reagent containing urea. Curve I, glucose; Curve II, mannose; Curve III, galactose; Curve IV, levulose.

In these reactions urea appears to play a part similar to that shown in the test of Nakashima and Maruaka. But it also

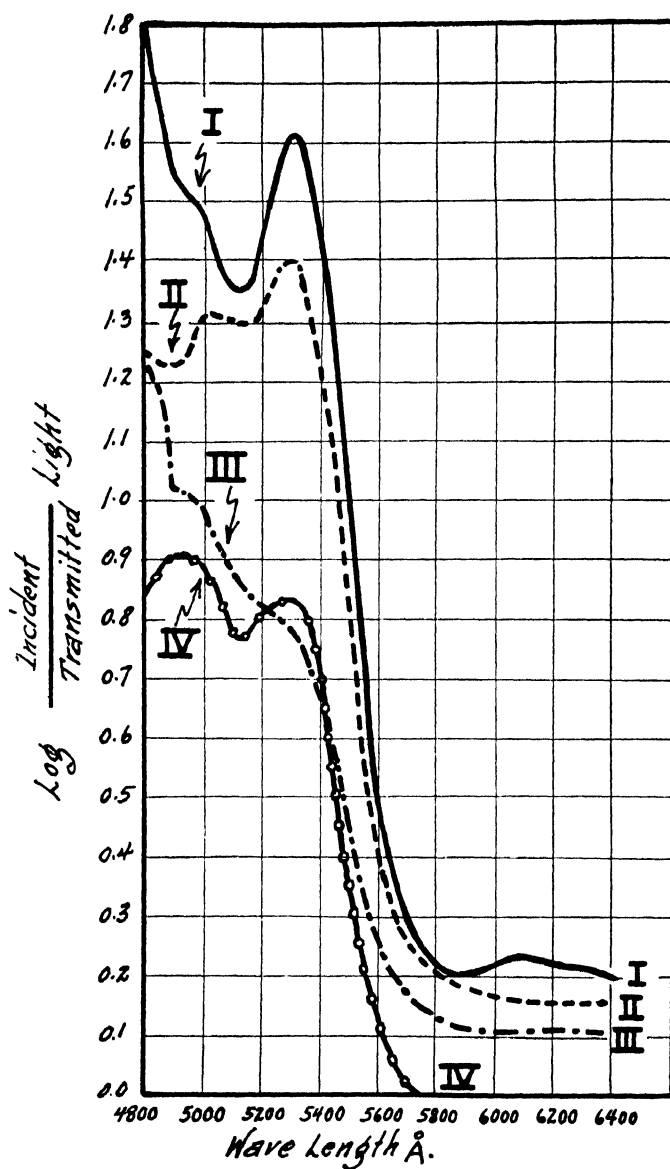


FIG. 2. Absorption spectra of color tests given with the reagent containing guanidine. Curve I, levulose; Curve II, glucose; Curve III, mannose; Curve IV, galactose.

subscribes in some way to the hue of the final color, for by substituting guanidine for urea one can obtain a quite different series of color reactions with the hexoses.

A reagent containing *guanidine* can be made by dissolving 25 gm. of guanidine in 80 cc. of 40 per cent (by volume) sulfuric acid, diluting to 100 cc. with acid, and saturating the solution with stannous chloride by long shaking with an excess of that substance. On standing, the undissolved stannous chloride separates and the clear supernatant fluid can be pipetted off as required.

3 cc. of the reagent with guanidine are boiled vigorously for 1 minute with 0.5 cc. of the sugar solution to be tested. With all the simple hexoses used (glucose, mannose, galactose, levulose) a color is just perceptible with a sugar concentration of 0.1 mg. in the 3.5 cc. reaction mixture. The colors obtained are quite different from those given by the reagent containing urea. There is no distinction between ketose and aldose reactions, but each sugar gives a color of its own. Levulose gives a distinctly red hue, mannose produces a red somewhat yellower than that given by levulose, the color from glucose is blue-red, while galactose gives a color bluer than that of levulose and mannose but yellower than that of glucose. The absorption spectra of these colors are shown in Fig. 2.

The pentoses and furfuraldehyde again give only a yellow test color.

The obvious difference between the results given by the reagent containing urea and that containing guanidine indicates that the chemical structure of both urea and guanidine enters into the reaction producing the colors.

#### SUMMARY

1. Two new reagents, containing, respectively, urea and guanidine dissolved in a stannous chloride-sulfuric acid solution, are found to give definite color reactions with the simple hexoses.

2. The reagent containing urea differentiates between ketohexoses and aldohexoses, the former giving a much more sensitive reaction and a bluer color.

3. The reagent containing guanidine gives a distinct color for each sugar without a definite distinction between ketohexoses and aldohexoses.



# THE PREPARATION OF OPTICALLY ACTIVE THIOHYDANTOINS AND THE RACEMIZATION OF AMINO ACIDS AS THEIR AZLACTONES

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A rather astonishingly mild and easy method for the racemization of optically active amino acids has been described by Bergmann and Zervas (1) and by du Vigneaud and Sealock (2). The observations reported in the present paper were the result of an attempt to throw more light on the mechanism of such racemizations.

It was first established that *d*-alanine is racemized substantially as are the other amino acids previously reported. When, however, *d*-alanine was heated (on a water bath) with 5 parts of acetic anhydride containing 10 per cent of glacial acetic acid, and somewhat more than 1 mol of ammonium thiocyanate,<sup>1</sup> the 1-acetyl-2-thio-5-methylhydantoin obtained was optically active,<sup>2</sup> though in other respects scarcely distinguishable from the racemic compound described by Johnson (5). In general, the numerous thiohydantoins which have been prepared have not been tested for optical activity. If optically active thiohydantoins prepared by this method have previously been recorded as such, the authors are not aware of the fact.

When the thiohydantoin preparation described above was carried out without the addition of the thiocyanate until after the first half hour of heating, the product isolated was completely inactive.

<sup>1</sup> Conditions for thiohydantoin formation (3-5).

<sup>2</sup> On account of its easy isolation and high specific rotation (+118.5° in 95 per cent alcoholic solution), this derivative should prove useful for the identification of active alanine.

Bergmann and Zervas (1) found that, while the first mol of acetic anhydride was without influence on an amino acid dissolved in glacial acetic acid, except for the formation of the N-acetyl derivative, the *second* mol of anhydride (or fraction thereof) acted catalytically to produce racemization. It was apparently from the catalytic nature of this process that they deduced that the azlactone could not be an intermediate in this process of racemization, since azlactone formation would destroy the fractional part of the second mol of anhydride, and therefore prevent the completion of the racemization. The latter process they assumed to take place in a hypothetical intermediate stage between the acetyl amino acid and the azlactone. On the basis, then, of their interpretation, none of their results would apply to the racemization of active azlactones, which is a subject on which no information is available, except perhaps the suspicion that active azlactones might be difficult to obtain, in view of the probability of racemization in the intermediate stage.

The only satisfactory interpretation that has been given for the reaction of thiohydantoin formation is that of Johnson and Scott (6), which definitely requires the formation of azlactone as an intermediate product. We have accordingly felt justified in using the isolation of a thiohydantoin as evidence that the corresponding azlactone had previously been formed. The results of the present investigation may then be interpreted as follows: (a) In the presence of a large excess of acetic anhydride, azlactone formation can take place so rapidly that little racemization occurs. (b) An *active azlactone* is thus formed. This can react with ammonium thiocyanate with sufficient rapidity to stabilize the product as the thiohydantoin with little racemization. (c) In the absence of thiocyanate, the *active azlactone*, which must have formed at approximately the same rate in both cases, is completely racemized in half an hour, and perhaps in less time, under the conditions used.

It is thus shown that optically active azlactones may be readily formed (although their *isolation* might perhaps present some difficulties); and that such azlactones are capable of racemization with an ease which at least suggests further study of the possibility that they may be responsible for the results obtained by Bergmann and Zervas (1) and by du Vigneaud and Sealock (2).

## EXPERIMENTAL

*d*-1-Acetyl-2-Thio-5-Methylhydantoin—A mixture of 1 gm. of *d*-alanine and 1 gm. of ammonium thiocyanate was heated for 0.5 hour on the water bath with 5 cc. of acetic anhydride which contained 10 per cent of acetic acid. When the solution was cooled and treated with 25 cc. of water, there apparently separated 1.0 gm. of the desired thiohydantoin. This material dissolved incompletely in 25 cc. of 70 per cent alcohol at room temperature, the undissolved part being chiefly inactive. The solution, treated with an equal volume of water and kept at 5°, deposited the practically pure *d* compound  $[\alpha]^{20} = 118.5^\circ$  in 1 per cent solution in alcohol for white light filtered through a 2 cm. cell of 3 per cent potassium dichromate solution. It melts at 161–162°. It is estimated that two-thirds of the thiohydantoin formed was optically active.

*Analysis*—(Kjeldahl). Calculated for  $C_6H_9O_2N_2S$ , N 16.27; found, N 16.21.

*dl*-1-Acetyl-2-Thio-5-Methylhydantoin (5)—The only variation from the method described above was that, in this case, the *d*-alanine was heated for 0.5 hour with the acetic anhydride-acetic acid mixture before the thiocyanate was added. Heating was then continued for an additional 0.5 hour. The product showed no trace of optical activity. It melted at 168–169°.

*Racemization of d-Alanine*—When *d*-alanine was heated on the water bath for 0.5 hour with glacial acetic acid containing 2 mols of acetic anhydride, no indication of optical activity could be found in the resulting product.

*Thiohydantoin Formation with Smaller Amounts of Anhydrides*—1 gm. each of *d*-alanine and ammonium thiocyanate was heated for 0.5 hour with 5 cc. of glacial acetic acid and 2.0 cc. (1.9 mols) of anhydride.<sup>3</sup> No product could be isolated until most of the acetic acid had been removed under reduced pressure and the resulting syrups treated with water. The yield was then 0.2 gm. of the thiohydantoin, of which 33 per cent was still unracemized.

<sup>3</sup> Aside from the fact that the usual excess of anhydride is lacking here, the proportion of anhydride to acetic acid is rather unsuitable for thiohydantoin formation (3).

The mother liquor from which this material was isolated was again evaporated to a syrup under reduced pressure and the residue condensed with ammonium thiocyanate under the usual favorable conditions. In this case 0.79 gm. of the thiohydantoin was isolated, and proved to be 70 per cent racemized.

These last experiments were considered to show that thiohydantoin formation and, therefore, azlactone formation could take place in the presence of less than 2 mols of acetic anhydride (1), and that even with this lower proportion of anhydrides the thiohydantoin formation offers considerable protection against racemization.

We believe that a derivative such as *d*-1-acetyl-2-thio-5-methylhydantoin, which can be prepared with ease and almost in quantitative yield, could serve with advantage in the (qualitative) identification of *d*-alanine. The report of Mr. George L. Keenan, micro analyst, Food and Drug Administration, describing the habit and the optical behavior of the crystals of *d*-1-acetyl-2-thio-5-methylhydantoin is as follows:

"In ordinary light, the substance is colorless, habit prismatic, usually various, and occasionally six-sided or elongated. The refractive indices as determined by the immersion method are  $\alpha = 1.485$ ,  $\beta = 1.685 (\pm 0.003)$ ,  $\gamma = > 1.733$ . Many fragments yield an intermediate value, 1.685, which closely approximates  $\beta$ . This value is shown by those fragments with the interference figure centered. In parallel polarized light, the double refraction is extremely strong. In convergent polarized light, the substance is biaxial, the two optic axes rarely appearing in the field simultaneously, but  $2E$  is indicated to be rather large. Partial biaxial interference figures, however, are common, frequently showing one optic axis in the field."

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## THE SYNTHESIS OF GLYCYLGLYCINE

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Glycylglycine has been synthesized by the following methods:

(1) glycine  $\rightarrow$  glycine ester hydrochloride  $\rightarrow$  glycine ester  $\rightarrow$  glycine anhydride  $\rightarrow$  glycylglycine; (2) glycine  $\rightarrow$  glycine acid chloride hydrochloride  $\rightarrow$  glycylglycine; (3) chloroacetic acid and ammonia  $\rightarrow$  glycylglycine (when the ammonia is removed by evaporation in the presence of calcium hydroxide); (4) glycine-toluenesulfoglycine hydrazide  $\rightarrow$  toluenesulfoglycylglycine  $\rightarrow$  glycylglycine; (5) glycine  $\rightarrow$  chloroacetyl glycine  $\rightarrow$  glycylglycine.

The first synthesis is well known and quite satisfactory. Glycine ester may be liberated from the hydrochloride by silver oxide (1, 2), sodium hydroxide (3), or barium hydroxide (4-6). Lead oxide (7) has been used for some ester hydrochlorides but it gives poor results with glycine. Glycine ester spontaneously decomposes to glycine anhydride (2) which is recrystallized to remove the contaminating biuret base (8, 9). Glycine anhydride has been prepared by heating glycine with glycerol (10). On hydrolysis with hydrochloric acid (11) or sodium hydroxide (12) glycine anhydride yields glycylglycine.

Method 2 was used by Fischer (13) for the preparation of a number of peptides but it appears that glycylglycine was not synthesized by this procedure. The hydrochloride of glycyl chloride was identified by the chlorine analysis and by its reaction with absolute alcohol to form the hydrochloride of glycine ester. Also later workers (14, 15) claim to have isolated glycyl chloride hydrochloride but no analytical proof is presented. In our work in which the conditions set forth by Fischer were followed with extreme care the product obtained in three experiments was shown by amino nitrogen and chloride analyses to be glycine hydrochloride.

The authors of Method 3 (16) have presented no convincing evidence that glycylglycine is actually produced.

The use of protective radicals in the synthesis of peptides has been extensively investigated. Curtius (17) and Fischer (18) found that the benzoyl and the carbethoxy groups could not be removed from the peptide without causing deep seated changes in the molecule. Abderhalden and Möller (19) have shown that certain other radicals retard the hydrolysis of the peptide and Schönheim (20) was able to prepare glycylglycine in about 90 per cent yield by heating its toluenesulfo derivative with hydriodic acid and phosphonium iodide (Method 4). These results are somewhat surprising since Colles and Gibson (21) obtained only 17 per cent hydrolysis of *p*-toluenesulfoalanine with constant boiling hydrochloric acid.

Method 5 has been used more than any other for the preparation of peptides although in the synthesis of glycylglycine only the intermediate chloroacetylglycine has been reported (22). A simplified preparation of glycylglycine is described in the present paper.

#### *Preparation of Glycylglycine*

50 gm. ( $\frac{2}{3}$  M) of crude glycine are dissolved in 670 cc. (half the theoretical amount) of N sodium hydroxide and the mixture cooled to 2–3°. 60 cc. (25 per cent excess) of freshly distilled chloroacetyl chloride and 750 cc. of N sodium hydroxide are added in alternate portions to the reaction mixture. The latter is stirred constantly and kept at about 5° by means of a freezing mixture. About 2 hours are required for these manipulations.

The solution is acidified with hydrochloric acid, 5 cc. excess of N hydrochloric acid added, and then it is evaporated to about 200 cc. The precipitated sodium chloride (about 35 gm.) is filtered on a Buchner funnel and the filtrate thoroughly mixed with 4 liters of 15 N ammonium hydroxide. After this solution has stood for about 18 hours it is evaporated to about 1 liter and then vacuum-distilled at about 40° to a residual volume of approximately 175 cc. The latter is mixed with 200 cc. of hot, 85 per cent methyl alcohol and filtered on a Buchner funnel. The filtrate is transferred to a beaker, mixed with 2 volumes of 95 per cent methyl alcohol, and then 300 cc. of diethyl ether are added slowly with stirring to the yellow, translucent solution to precipitate the crude glycylglycine

as a dark, yellow oil which changes rapidly to a pasty mass. After standing for an hour the ether-alcohol layer is decanted and the solid cake is ground in a mortar and dissolved, by careful heating, in about 75 cc. of distilled water. 10 volumes of 95 per cent methyl alcohol are added and, after standing in the ice box for 5 or 6 hours, the precipitated glycylglycine is filtered. After about four crystallizations the peptide is obtained as a tasteless, white solid free from chloride ions. The yield is about 45 per cent of theory. As described by Fischer and Fourné (11) it crystallizes in small tetrahedric leaves with a lustrous ball in the center.

The melting point is given by Fischer and Fourné as 215–220° with browning and by Siegfried (23) as 235–236° (corrected). When determined by the usual capillary tube method our product browns at 202° and is charred at 221° but the true decomposition point is believed to be 260–262°. The latter value was obtained by a new procedure described in the following paper.

*Analysis*—Calculated for  $C_4H_8O_3N_2$ , N 21.2; found (Kjeldahl) 21.0, (Van Slyke) 14.5, 14.5.

The latter figures are 136.8 per cent of the theoretical value (10.6 per cent). The abnormalities previously reported (20, 24, 25) range from 125 to 138 per cent.

*Addendum*—Since this paper was sent to press we find that Freudenberg (26) has obtained glycylglycine in good yield by hydrolyzing glycine anhydride with 10 per cent ammonia in a pressure flask. Bergman and Zervas (27) have utilized the carbobenzoxy radical for the synthesis of dipeptides although glycylglycine was not prepared.

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## DECOMPOSITION POINTS OF THE AMINO ACIDS

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It is well known that the melting points reported for crystalline substances which decompose are unreliable. This is illustrated by the amino acids, particularly glycine and alanine, for which values over a wide range have been found by different authors (see Table I).

The need for more certain identification of such substances has been met indirectly by the use of a mixed solubility procedure (1, 2) and a boiling point rise method (3). However, Dennis and Shelton (4) have shown that sharp melting points can be obtained with substances which decompose when their apparatus, consisting of an electrically heated copper bar, a constantan-copper thermoelectric pair, and a potentiometer, is used.

Low values are obtained by conventional methods because the sample is exposed to the heat of the bath as the temperature is raised. Although this effect may be minimized by rapid heating of the bath or by heating it to approximately the melting temperature of the substance before introducing the sample, the inherent error is not entirely eliminated. By shortening the preheating time to a few seconds Dennis and Shelton have overcome this difficulty to a considerable extent. In view of these relations it occurred to us that it might be possible to establish true melting (or decomposition) points for the amino acids if a procedure could be devised for producing this change in physical state almost instantly. Such a method, described in this paper, consists simply of a precise estimation of the time and temperature required for the amino acid to reach a standard decomposition state as judged by the shade of yellow or brown color produced. Then the time-temperature values are plotted and the melting (or decomposition)

point of the substance is considered to be that temperature at which the curve becomes parallel to the base axis. At this point the position of the curve also is a measure of the thermal lag; *i.e.*, the time required to heat the capillary tube to the temperature of the bath.

It was found by repeated experiments that a standard color could easily be reproduced to  $\pm 0.1$  second and  $\pm 0.1^\circ$  and that identical decomposition temperatures were obtained by individuals working independently. Also, it was shown that identical decomposition values for a given amino acid were indicated when different, standard decomposition colors were used.

#### EXPERIMENTAL

The apparatus (see Fig. 1) used for determining melting (or decomposition) points is similar to that described by Beattie and Jacobus (5). The iron vessel, 4 inches in diameter and 4 inches deep, was wound with 16 gage, chromel wire and surrounded with asbestos. A mixture of 30 per cent lead and 70 per cent tin, fluid at temperatures above  $185^\circ$ , was used to fill the iron vessel within  $\frac{3}{4}$  inch of the top.

The capillary tube, containing the amino acid sample, was attached in a shallow groove to an iron rod,  $\frac{1}{4}$  inch in diameter and 7 inches long. When an experiment was being carried out this rod was permitted to fall freely into the bath through an opening in the iron cover. In this lowered position the end of the rod was flush with the cover and the capillary tube was immersed to a constant depth in the bath. The mechanical arrangements were such that the time during which the capillary tube was immersed in the bath was automatically measured to 0.1 second.

The thermopile consisted of nineteen iron-constantan couples, each joined together with silver solder. Each couple was enclosed at the hot junction in supertite (porcelain) insulators and at the cold junction in 3 mm. spaghetti. The special iron (24 gage) and constantan (30 gage) wire were purchased from Charles Engelhard, Inc., Newark. The thermopile, inclosed at each end in a glass tube and connected to a Leeds and Northrup student potentiometer, was calibrated against standard substances. When the voltages obtained (see Table II) were plotted against

the corresponding temperatures, the curve was nearly a straight line.

The soft glass, capillary tubes were cut to a standard length of 6.5 cm. and sorted into three sizes according to diameter. The thick tubes weighed  $0.053 \pm 0.004$  gm., the medium ones  $0.034 \pm 0.004$  gm., and the thin ones  $0.023 \pm 0.004$  gm. Since the thermal lag was different for each group, only tubes of the same diameter

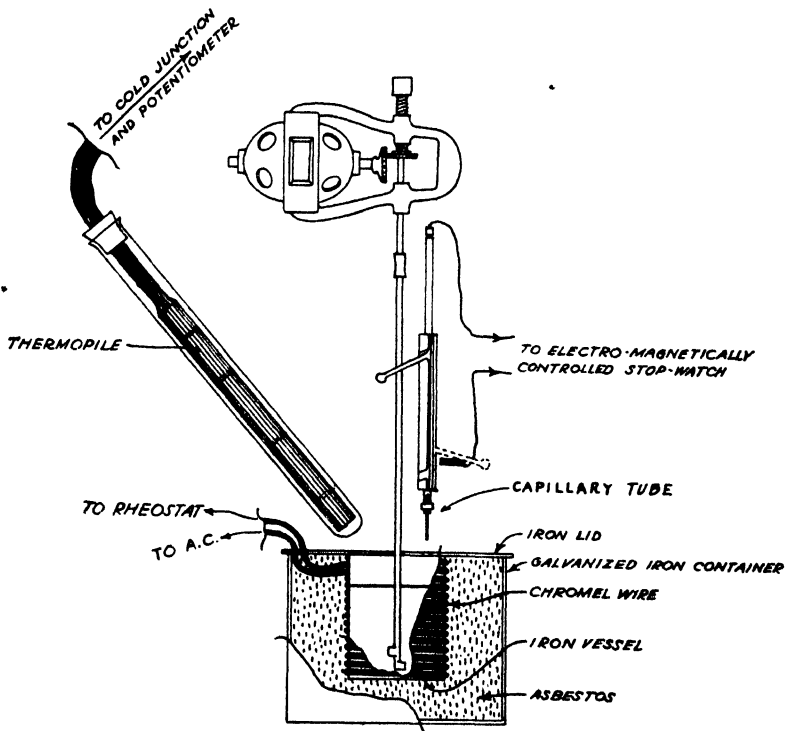


FIG. 1. Apparatus used for determining melting points

were used for establishing a complete decomposition curve. Each tube was filled to a depth of 10 mm. with finely ground amino acid.

The amino acids used in this work were all synthetic samples prepared in this laboratory by standard methods except for *d*-glutamic acid and *l*-tyrosine which were isolated from natural sources and 3,5-diiodo-*l*-tyrosine and *l*-leucine which were obtained from the S. M. A. Corporation, Cleveland. With the exception of the last

## 224      Decomposition Points of Amino Acids

TABLE I

*Decomposition Points in °C. of Amino Acids and Peptides*

The figures in parentheses indicate bibliographic citations; br., the temperature at which the brown color is developed; unc., uncorrected; and cor., corrected.

### Glycine

- 289-292 (authors)
- 290.3 (Dennis and Shelton)
- 200 (br.), 225-230 (unc.) (6)
- 225-230 (7)
- 228 (br.), 232-236 (8)
- 228 ( " ), 236-239 (unc.) (9)
- 212-240 (plates) (10)
- 222-241 (needles) (10)
- 232 (11)
- 232-236 (12)
- 230 (cor.) (br.), 238 (cor.) (13)
- 240-256 (unc.) (14)
- 247 (cor.) (15)
- 262 (16)

### *d*-Glutamic acid

- 247-249 (authors)
- 255.2 (Dennis and Shelton)
- 202-202.5 (17)
- 206 (cor.) (18)
- 213 ( " ) (19)

### *dl*-Glutamic acid

- 225-227 (authors)
- 185 (cor.) (20)
- 193.5-194.5 (cor.) (21)
- 195-196 (22)
- 198 (cor.) (23)
- 199 ( " ) (18)

### *l*-Tyrosine

- 342-344 (authors)
- 336 (Dennis and Shelton)
- 235 (unc.) (24)
- 265 ( " ) (closed tube) (25)
- 272 (open tube) (25)
- 287 (26)
- 289 (27)
- 290-295 (28)
- 295 (29)
- 310-314 (cor.) (30)
- 314-318 ( " ) (31)

### *dl*-Alanine

- 297 (authors)
- 258 (sublimes); did not melt at 390 (Dennis and Shelton)
- 195 (32)
- 228 (br.), 268 (plates) (10)
- 240 ( " ), 270 (needles) (10)
- 250 (33)
- 264-265 (closed tube) (34)
- 264-266 (cor.) (35)
- 280 (36)
- 290 (cor.) (37)
- 293 (38)
- 295 (cor.) (39)

### *dl*-Phenylalanine

- 318-320 (authors)
- 305.8 (Dennis and Shelton)
- 231 (40)
- 256 (41)
- 260 (42)
- 260-262 (43)
- 262 (44)
- 261-264 (45)
- 263 (46)
- 263-264 (unc.) (47)
- 263-265 (28)
- 271-273 (cor.) (48)

### *dl*-Aspartic acid

- 278-280 (authors)
- 328 (Dennis and Shelton)
- 140-150 (closed tube) (49)
- Above 300 (50)
- 325 (br.), 370 (51)

### 3,5-Diiodo-*l*-tyrosine

- 239-241 (authors)
- 204 (unc.) (52)
- 196-205 (53)
- 213 (cor.) (54)

TABLE I—*Concluded*

Glycylglycine	<i>dl</i> -Isoleucine
262-264 (authors)	292 (authors)
215-220 (55)	183 (sublimes); did not melt at
236-236.5 (cor.) (56)	390 (Dennis and Shelton)
Glycylglycylglycine	275 (closed tube) (64)
262-265 (authors)	<i>dl</i> -Norleucine
215 (br.), 246 (cor.) (57)	327 (authors)
<i>l</i> -Leucine	207 (sublimes); did not melt at
337 (authors)	390 (Dennis and Shelton)
291 (unc.) (58)	170 (sublimes) (65)
293-295 (59)	294-296 (66)
<i>dl</i> -Leucine	297-300 (closed tube) (67)
332 (authors)	<i>dl</i> -Valine
222 (sublimes); did not melt at	292 (authors)
390 (Dennis and Shelton)	190 (sublimes); did not melt at
270 (closed tube) (60)	390 (Dennis and Shelton)
290-291 (61)	298 (cor.) (closed tube) (68)
287-290 (cor.) (62)	
290 (63)	
293-295 (cor.) (59)	

TABLE II  
*Calibration Data for Iron-Constantan Thermopile*

Substance	Pressure (corrected)	Boiling point (corrected)	E.M.F.
	<i>mm.</i>	<i>°C.</i>	<i>volt</i>
Aniline . . . . .	757.5	183.8	0.1847
Naphthalene . . . . .	757.5	217.8	0.2205
Diphenylamine . . . . .	757.5	301.8	0.3054
Benzophenone . . . . .	746.0	305.1	0.3092
Sulfur . . . . .	746.0	443.9	0.4444

two, all substances were carefully purified by four or more crystallizations from water or water and alcohol and then dried thoroughly in an oven or a desiccator.

## DISCUSSION

Since the melting point of only one amino acid, glycine, was determined by Dennis and Shelton (4) it was of interest to us to obtain data on highly purified samples of this and other amino acids. Accordingly such samples were sent to Professor Dennis and Mr. Shel-

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ton to whom we wish to express our appreciation for making the determinations listed in Table I. A chromium-plated, copper bar was used in this work.

The melting points usually found for the amino acids are without fundamental significance since they vary according to the technique of the observer. For example, in the case of glycine a melting range of 232–236° seems to have been most generally accepted although figures from 10–30° higher have been reported (14–16).

TABLE III  
*Time-Temperature Relations for Standard Decomposition Colors of Glycine*

Light color			Medium color			Dark color		
Time	EMF	Temperature	Time	EMF	Temperature	Time	EMF	Temperature
<i>sec</i>	<i>mv</i>	<i>°C</i>	<i>sec</i>	<i>mv</i>	<i>°C</i>	<i>sec</i>	<i>mv</i>	<i>°C</i>
30 0	234 5	231 5	40 0	234 5	231 5	55 0	234 5	231 5
12 5	245 0	242 0	20 0	245 0	242 0	22 5	245 0	242 0
6 0	255 0	252 0	10 0	255 0	252 0	11 0	255 0	252 0
4 5	260 0	257 0	7 5	260 0	257 0	8 5	260 0	257 0
3 0	265 0	262 0	5 0	265 0	262 0	6 4	265 0	262 0
2 5	270 0	267 0	3 7	270 0	267 0	5 1	270 0	267 0
1 9	275 0	272 0	3 2	275 0	272 0	4 3	275 0	272 0
			2 6	280 0	277 0	3 5	280 0	277 0
1 7	285 0	282 0	2 2	285 0	282 0			
			2 1	287 2	284 2			
1 4	290 0	287 0	1 6	290 0	287 0	1 9	290 0	287 0
1 3	292 0	289 0	1 4	292 0	289 0	1 6	292 0	289 0
1 2	295 0	292 0	1 4	295 0	292 0	1 5	295 0	292 0

In our preliminary experiments a faint decomposition color was noted when glycine was heated for 5 minutes at 200° while decomposition appeared to be complete after 15 minutes at this temperature. Also no perceptible decomposition occurred when glycine was heated for 10 seconds at 235° while at 290° it was practically complete in 2 seconds. That the latter is the approximate true decomposition temperature was confirmed by the further experiments of Dennis and Shelton and the present authors (see Table III).

In general the data obtained with the Dennis-Shelton apparatus seem to be more trustworthy than any previously reported but,

with the exception of glycine, *d*-glutamic acid, and *dl*-aspartic acid, these too appear to be low. According to our experience sharp melting points are not easily obtainable and it is difficult to measure the melting (or decomposition) temperature with an accuracy greater than 2–3°. Furthermore, the physical change undergone when an amino acid is heated even under almost ideal conditions seems to be a decomposition although it may be accompanied by melting or dissolving of the decomposition products. Hence it is probably more satisfactory to classify this phenomenon as decomposition rather than melting.

In examining fifteen amino acids and peptides five different phenomena were observed during the heating. The five groups are: (1) substances which give off water as a decomposition product and then liquefy (*d*-glutamic acid, *dl*-glutamic acid, and *dl*-phenylalanine), (2) substances which give brown decomposition products (glycine, glycylglycine, and glycylglycylglycine), (3) substances which have yellow decomposition products (*l*-tyrosine and 3,5-diiodo-*l*-tyrosine), (4) substances which sublime (*l*-leucine, *dl*-leucine, *dl*-isoleucine, *dl*-norleucine, *dl*-valine, and *dl*-alanine), and (5) one substance which liquefies, decomposes, and then resolidifies (*dl*-aspartic acid). The decomposition point of substances in Groups 1, 4, and 5 was considered to be that temperature at which the sample became fluid. Capillary tubes, 3 cm. long and closed at both ends, were used for substances which sublime in an open tube.

Two experiments to determine the effect of added impurity on the decomposition point of glycine were performed. It was found that glycine with 10.7 per cent ammonium chloride impurity decomposed at 247–249° and with 1.4 per cent at 284–286°.

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## VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS

### III. INABILITY OF THE RAT ORGANISM TO SYNTHESIZE THE ESSENTIAL UNSATURATED FATTY ACIDS\*

BY HERBERT M. EVANS AND SAMUEL LEPKOVSKY

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(Received for publication, September 28, 1932)

As will be shown in the present communication, when rats are reared on complete diets, their body fats can be shown by direct feeding tests to contain appreciable amounts of the essential unsaturated fatty acids (vitamin F). As will also be shown, the vitally necessary substance is, however, either absent or greatly reduced in the carcass fat of rats reared on fat-free diets.

To determine this, fats were isolated from the carcasses of rats maintained on different types of diets. Three diets<sup>1</sup> were used, stock Diets I and XIV and fat-free Diet 616. Adult female rats raised on these diets were ground up, their fats extracted with hot ethyl alcohol and ether, and converted to the methyl esters.

\* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the Rockefeller Foundation, and from the Bureau of Dairying of the United States Department of Agriculture. These funds have been generously augmented by the Board of Research and the College of Agriculture of the University of California.

<sup>1</sup> The compositions of the diets cited are as follows: *Diet I*—Whole wheat, 67.5; casein, 15; whole milk powder, 10; sodium chloride, 1; calcium carbonate, 1.5; milk fat, 5. *Diet XIV*—Whole wheat, 67.0; fish oil, 5; casein, 5; alfalfa, 10; fish meal, 10. Diets I and XIV are supplemented twice weekly with fresh lettuce. *Diet 616*—Casein (Van Slyke, extracted 1 week with ether), 24.0; sugar, 72.1; Salt Mixture 185 (1), 3.9 supplemented with 1.0 gm. of ether-extracted yeast; non-saponifiable matter from cod liver oil (2), equivalent to 83 mg. daily; the non-saponifiable matter from wheat germ oil equivalent to 500 mg. weekly (3). We wish to take this opportunity to thank the F. E. Booth Company of San Francisco for their generous contribution of the fish oil and fish meal used in these experiments.

There is always a small per cent of rats less severely distressed than the others on Diet 616, and such rats were used as the source of the methyl esters in this experiment.

To determine whether on the day of weaning (when the experimental diet was begun) rats normally contain the essential unsaturated fatty acids, the fat from such rats was studied. The iodine numbers of the methyl esters of rat fat studied are listed in Table I.

The methyl esters were fed in quantities of 5 drops daily to rats which had been reared on fat-free diets devoid of essential unsaturated fatty acids. At the time of feeding, these rats showed the characteristic symptoms (3, 4) due to this deficiency. The results obtained when the methyl esters were fed are shown in

TABLE I  
*Iodine Numbers of Methyl Esters of Rat Fat*

Type of anima	Iodine No.
21 day-old females from mothers on stock Diet I.....	50.2
Adult females, stock Diet XIV.....	100.0
“ “ “ “ I.....	80.0
“ “ Diet 616, Lot I.....	69.0
“ “ “ 616, “ II.....	71.0

Figs. 1 and 2. The growth during the 30 day period previous to the feeding is indicated by the heavy line while the light line indicates the period during which the animals received the methyl esters. The small circles interrupting the curves indicate the occurrence of estrus.

It is seen that the fats from 21 day-old females (just weaned) contained the curative unsaturated fatty acids. Likewise the fats from the rats on the stock diets, Diets I and XIV, contained an abundance of these materials. However, the fat from the rats on the fat-free diet (Diet 616) did not contain such curative fatty acids (Fig. 2). Two different lots (Lots I and II) of the methyl esters of rats fed fat-free diets were prepared and fed at different times; the responses in each case were similar.

When the responses obtained with the various methyl esters are compared with the iodine numbers (Table I) it will be noticed

that the degree of unsaturation is not a criterion for the content of the curative fatty acids. Thus, the rats fed fat-free diets have body fats with more unsaturated fatty acids than rats at the

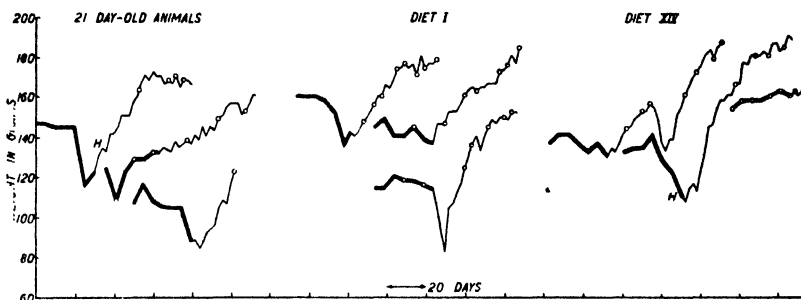


FIG. 1. Curves illustrating the responses obtained when the methyl esters of animals reared on the stock diets (Diets I and XIV) were fed to animals reared on the fat-free diet (Diet 616). The growth during the 30 day period previous to the feeding is indicated by the heavy line; the period during which the animals received the methyl esters, by the light line. The small circles interrupting the curves indicate the occurrence of estrus. The methyl esters from 21 day-old animals are likewise presented. *H* indicates hematuria.

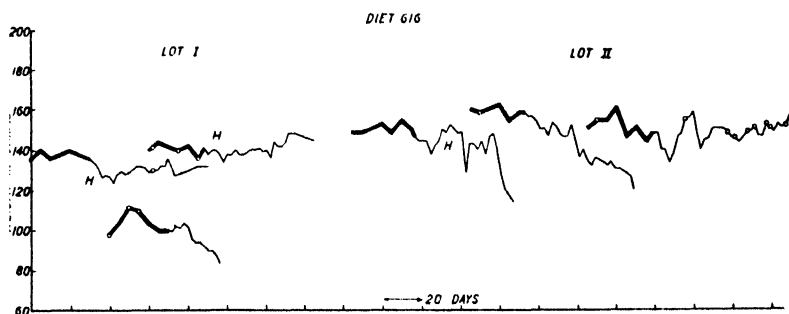


FIG. 2. Curves illustrating the responses obtained when the methyl esters of animals on the fat-free diet (Diet 616) were fed to animals reared on the fat-free diet (Diet 616). The growth during the 30 day period previous to the feeding is indicated by the heavy line; the period during which the animals received the methyl esters, by the light line. The small circles interrupting the curves indicate the occurrence of estrus. Lot I prepared and fed December, 1930. Lot II prepared and fed April, 1931. *H* indicates hematuria.

time of weaning, yet they possess smaller amounts of the curative unsaturated fatty acids.

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## THE SPARING ACTION OF FAT ON VITAMIN B

### IV. IS IT NECESSARY FOR FAT TO INTERACT WITH VITAMIN B IN THE ALIMENTARY CANAL TO EXERT ITS SPARING EFFECT?\*

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(Received for publication, September 28, 1932)

In our studies on the influence of fat upon the vitamin B (1) requirements of the rat, the question arose as to whether the beneficial effects of the fat could in any way be the result of the interaction of fat with the vitamin B in the alimentary canal.

The answer to this question was sought in the parenteral administration of either vitamin B or the fat or both. To date, attempts to administer parenterally fats in sufficient quantities to exert a definite sparing action has met with failure. However, it is possible to administer vitamin B parenterally, and we desire in this note to present evidence which shows that fat in the diet spares vitamin B when inadequate amounts of this vitamin are given intraperitoneally. Litter mate sisters were reared on either a fat-free diet or one which contained 25 per cent cottonseed oil. The vitamin B was administered by mouth or by intraperitoneal injection. Two levels of vitamin B were fed—one which is inadequate for growth and one which with fat in the diet produces normal growth. The composite growth curves of these four groups of animals are shown in Fig. 1. It will be seen that the addition of fat to the diet caused practically the same improvement of growth in the animals receiving their vitamin B intake parenterally as was the case when vitamin B was fed. When other factors are the same, a slight but consistently poorer growth

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is always shown by animals receiving vitamin B parenterally as contrasted with oral administration. Our results definitely demonstrate that the sparing action of fat on vitamin B cannot be ascribed to an interaction in the alimentary canal between these two substances.

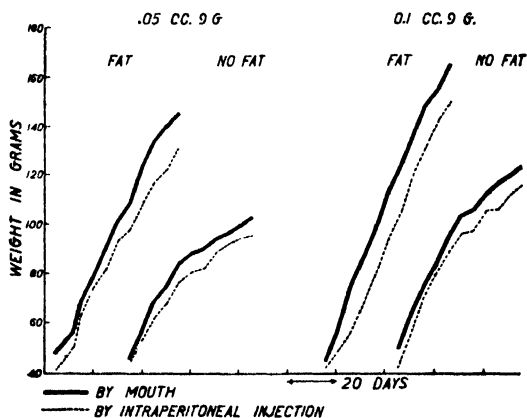


FIG. 1. Composite growth curves of six animals each. Extract 9-G is prepared by extracting rice polishings with 25 per cent alcohol, concentrating to a syrup, adding 95 per cent alcohol until the concentration is 80 per cent, filtering, and concentrating the extract (2).

	Fat	No. fat
Casein (L-3) (1).....	30.0	20.0
Sugar.....	41.0	70.0
Autoclaved yeast*.....	10.0	10.0
Salt Mixture 185 (3).....	4.0	4.0
Cottonseed oil.....	25.0	

Supplemented with 2 drops of cod liver oil daily.

\* The whole dried yeast was generously supplied by the Fleischmann Laboratories of Standard Brands Incorporated.

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## THE SPARING ACTION OF FAT ON VITAMIN B

### V. THE RÔLE OF GLYCERIDES OF OLEIC ACID\*

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(Received for publication, September 28, 1932)

We have already reported studies with the glycerides of single saturated fatty acids (1) with reference to their ability to spare vitamin B. We chose as the next step the investigation of oleic acid as the simplest unsaturated fatty acid of biological importance. Both commercial and highly purified oleic acids were fed as the glycerides.

#### *Experimental Procedure*

Commercial oleic acid was obtained on the market and distilled *in vacuo* at 170–190°. It then had an iodine number of 85 to 86, indicating it was largely oleic acid, though it undoubtedly contained some saturated fatty acids as well as linoleic acid.

Purified oleic acid was prepared by treating the lead soaps of olive oil with alcohol (2) and ether (3), and the barium soaps with equal parts of 95 per cent ethyl alcohol and dry benzene (4). One of the criteria of purity used in these studies was the degree to which one can continue to crystallize, from salts of oleic acid, the salts of other fatty acids. Such data combined with iodine values are naturally more reliable than iodine values alone, for mixtures of palmitic and linoleic acids could, for instance, give an iodine value identical with that of pure oleic acid.

The fatty acids were esterified with glycerol at 200–230°. A

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stream of dry CO<sub>2</sub> constantly agitated the mixture, and at the same time aided in the removal of water formed in the reaction. The glycerides were fed as 25 per cent of the diet.<sup>1</sup> In all cases where the feeding of fatty acids is mentioned, the glycerides are, of course, meant. Three levels of vitamin B were used: no yeast, 50 mg. of yeast, and 1 gm. of yeast.

In an attempt to simulate a natural fat, 40 parts of the glyceride of stearic acid were mixed with 60 parts of the glyceride of oleic acid prepared from the commercial oleic acid. The stearic acid used was similar to that employed previously (1). It gave inferior growth since only about one-half of the ingested stearic acid was absorbed. The product was not pure and was probably contaminated with oleic and palmitic acids.

*Results with Commercial Oleic Acid Alone and When Mixed with Stearic Acid*

Fig. 1 illustrates the results obtained with commercial oleic acid and stearic acid alone and with the mixture of the two. Oleic acid alone with inadequate vitamin B is inferior to the fat-free diet.<sup>2</sup> So is the diet containing the stearic acid. The inferiority due to stearic acid can be explained by the difficulty rats have in absorbing it, about 50 per cent escaping unabsorbed (1). No such explanation is available for the inferiority of commercial oleic acid. Under the same conditions where either stearic acid or oleic acid fed separately is inferior, the mixture, 40 parts of stearic acid and 60 parts of oleic acid, is definitely superior.

With an adequate level of vitamin B, the results with commercial oleic acid are different. There is no inferiority evident when compared with the fat-free diet. The mixture of stearic and oleic acids is very little superior to the oleic acid alone. The stearic acid by itself is somewhat inferior, due to difficulty in absorption.

*Results with Purified Oleic Acid*

Fig. 2 illustrates the results with purified oleic acid. Commercial oleic acid administration was repeated in this series for

<sup>1</sup> The general formula for the diets containing the glycerides is: casein (L-3) (1), 30.0; Salt Mixture 185 (5), 4.0; sugar, 41.0; autoclaved yeast, 10.0; *glyceride*, 25.0.

<sup>2</sup> Fat-free diet: casein (L-3), 20.0; autoclaved yeast, 10.0; sugar, 70.0; Salt Mixture 185, 4.0. Supplemented with 2 drops of cod liver oil daily.

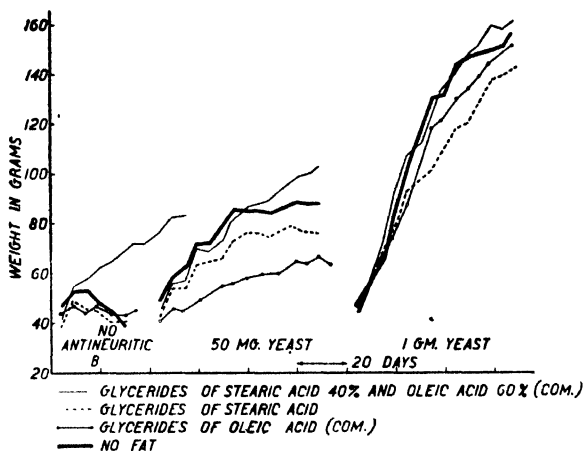


FIG. 1. Composite curves depicting the growth obtained when commercial oleic acid and stearic acid are fed separately and in a mixture. Each curve represents the average growth of four animals.

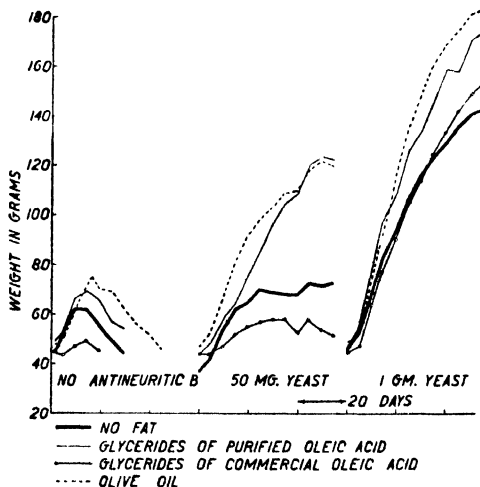


FIG. 2. Composite curves depicting the results obtained with commercial oleic acid and with purified oleic acid. Each curve represents the average growth of six animals.

purposes of comparison. Diets containing olive oil and fat-free diets were fed as controls.

Here it is at once evident that when vitamin B is inadequate, purified oleic acid yields results radically different from those obtained with commercial oleic acid. Whereas the results with commercial oleic acid show inferiority to fat-free diets, the results with purified oleic acid are superior to fat-free diets, showing definite sparing action.

When vitamin B is adequate, the results with commercial oleic acid equal those with the fat-free diet and are almost as good as with purified oleic acid. The olive oil behaves as other natural fats (6) in the superior growth it promotes. It is interesting that the purified oleic acid gives similar results.

#### SUMMARY

1. Glycerides of purified oleic acid spare vitamin B to about the same degree as natural fats.

2. Glycerides of commercial oleic acid give results decidedly inferior to the glycerides of purified oleic acid when vitamin B is inadequate. When vitamin B is adequate the glycerides of commercial oleic acid are almost as good as the glycerides of purified oleic acid.

3. Growth obtained with mixtures of the glycerides of commercial oleic and stearic acids is superior to that obtained when the glyceride of either fatty acid is fed alone.

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## ENZYMES OF THE MAMMARY GLAND\*

### THE PRESENCE OF GLUCOMALTASE IN THE MAMMARY GLAND

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#### INTRODUCTION

The question of the mechanism of the origin of the various constituents of milk is a very interesting one and several authors have undertaken the study of it. Bradley (1) (1912-13) examined the mammary gland for the enzymes, lactase and lipase, on the theory that these enzymes, by reversible action, should be able to effect a synthesis of lactose and fat, respectively. However, he was unable to find any lactase and contrary to the findings of Loevenhart (2), not enough lipase to account for the synthesis of fat. Grimmer (3) about the same time reported his findings regarding a great number of enzymes in mammary tissue such as: proteases, lipase, amylase, and salolase, all of which he stated were present in the gland. He did not test, however, for lactase or other invertases. According to Roehman's theory (4) there should be a lactose-synthesizing enzyme in the mammary gland, with the ability to convert glucose into galactose and to combine the two monoses then to lactose; but he did not furnish proof for his statement. He also thinks that there are stereokinases in the mammary gland which convert sucrose into lactose. Hesse (5) of the same laboratory believes that maltose may be an intermediary product of this process since he prepared maltosazone from a digest of macerated glands. Virtanen (6), in a more recent paper, studied the lipase content of the mammary gland and states that there is only a trace of lipase present. This could be detected

\* This work was aided by a grant from the Committee on Scientific Research, American Medical Association.

only after continuous extraction of the gland tissue with glycerol for 42 days.

We have examined the mammary gland for the enzymes, amylase, maltase, sucrase, lactase, lipase, salolase, proteases, and stereokinases, by modern methods, *viz.* control of pH, control of buffer action on substrates, etc. The earlier work obviously was not carried out under these conditions.

#### EXPERIMENTAL

*Preparation of Extract*—Cow mammary glands were obtained from the slaughter-house, immediately freed from fat mechanically, and from blood by washing with cold water, and were ground in a meat chopper. 1 kilo of ground tissue was extracted with 1.5 liters of glycerol for 4 weeks at room temperature and filtered. The pH of the filtrate of the glycerol extract after 4 weeks extraction was 5.9.

*Purification of Glycerol Extract*—500 cc. of the glycerol extract were dialyzed in collodion bags against distilled water for 2 days to get rid of reducing substances, protein decomposition products, and the glycerol. The precipitate which formed in the dialyzing bag was suspended in 200 cc. of distilled water. This milky suspension was found to be several times more active than the original extract, when tested for maltase activity. In all experiments to be described, this purified extract, as well as the original glycerol extract, was used.

*Experiment I. Test for Proteases*—Since all proteases clot milk and since this property is the most sensitive of all the characteristics of proteases, we used the clotting of milk by our method (7) as a criterion for proteolytic activity. As a substrate  $\text{CaCl}_2$ -milk was employed. No clotting could be noticed after 2 days incubation at  $37^\circ$ . Toluene was used in this as well as in all other experiments as an antiseptic.

*Experiment II. Test for Lipase*—To 10 cc. of the glycerol extract 90 cc. of distilled water were added. The pH of this solution was 6.0. To 50 cc. of this diluted solution 1.5 cc. of ethyl butyrate and 2 cc. of toluene were added and incubated at  $37^\circ$ . After 24 hours an aliquot sample was titrated with 0.1 N NaOH, litmus being used as an indicator. There was found only a very slight lipolytic

activity (increase in acidity). Similar results were obtained at other pH's (7.0 and 8.5) and with other dilutions.

*Experiment III. Test for Salolase. Effect of Original Glycerol Extract on Salolase*—To 5 cc. of the glycerol extract of pH 5.9, 0.2 gm. of salol was added. After 24 hours incubation at 37° the proteins were precipitated with 45 cc. of 95 per cent ethyl alcohol, filtered, and to 5 cc. of the filtrate 2 drops of 1 per cent  $\text{FeCl}_3$  were added. A light brown color resulted. A control, containing glycerol instead of the glycerol extract, yielded a light brown color exactly equal to the first in intensity. This shows that there is no salolase in the mammary gland and that only a very slight hydrolysis took place due to the action of the buffer. (Salolase liberates phenol from salol, giving an amethyst color with  $\text{FeCl}_3$ ; traces of phenol may give a slight brownish color.)

*Experiment IV. Test for Lactase*<sup>1</sup>—To 3.2 cc. of the extract 0.8 cc. of 0.1 N acetate buffer of pH 4.4 and to two other similar samples 1 cc. of each of a buffer of pH 6.6 and 7.0, respectively, were added. To each of the series 1 cc. of 1.42 per cent lactose was added. 1 cc. samples were taken of the digests and 4 cc. of 95 per cent ethyl alcohol were added to precipitate the proteins. In the filtrate hydrolysis was determined by the authors' monose method (8). There was no lactase activity noticeable during 14 days of incubation at 30° in any of the samples.

*Experiment V. Test for Lactose-Synthesizing Power*<sup>1</sup>—To a mixture of 1 cc. of 1.42 per cent glucose and 1.42 per cent of galactose, 2.2 cc. of the extract and 0.8 cc. of each of buffers of pH 4.4, 6.6, and 7.0, respectively, were added. There was no decrease in reducing power during 2 weeks at 30°.

*Experiment VI. Test for Amylase*—To 8 cc. of the extract 1 cc. of 0.1 N acetate buffer of pH 6.8 and 2 cc. of a 1 per cent starch paste were added. Controls, containing either no starch, no extract, or no buffer, respectively, were also run. The increase in reducing power was determined in 1 cc. of the digest by precipitating the proteins and the starch with 4 cc. of 95 per cent ethyl alcohol and using 1 cc. of the filtrate for analysis by the new copper method of Benedict (9). After 24 hours there was only a slight increase in the reducing power of the first sample, which was equal

<sup>1</sup> These experiments were also carried out on aqueous, on 33 per cent acetone, and on 33 per cent alcoholic extracts of the mammary gland.

to that of the buffered sample of starch. The temperature was 30°.

*Experiment VII. Test for Maltase*—To 2 cc. of the extract 1 cc. of 1.42 per cent maltose and 1 cc. of distilled water were added. After 1 day, 51 per cent of the maltose was hydrolyzed; after 2 days, 74 per cent; and after 3 days, 85 per cent.

*Controls*—(a) To 2 cc. of glycerol, 1 cc. of buffer of pH 5.9 (0.1 N acetate) and 1 cc. of 1 per cent maltose were added. After 3 days, 12 per cent of the maltose was hydrolyzed. (b) A boiled sample of glycerol extract to which maltose was added showed only a very slight reduction when immediately tested (because of reducing substances present in the extract). The incubation temperature was 30°. The degree of hydrolysis was determined by adding to 1 cc. of the digest 4 cc. of alcohol to precipitate the proteins and then 1 cc. of the filtrate was used for analysis by the authors' monose method (8).

*Experiment VIII. Other Experiments on Maltase. Sucrase Activity*—These experiments were carried out at pH 4.5 and 6.0. Other conditions were the same as those used for the test for maltase activity. A substrate of 1.42 per cent sucrose and one of 4 per cent were used. No reduction was noticeable after 3 days incubation at 30°. This shows that neither the extract nor the maltase possesses sucrase activity and that there is no stereokinase in the mammary gland, capable of transforming sucrose into a reducing biose.

*Glucosidomaltase Activity*—To each of three tubes 2 cc. of extract, 1 cc. of a 2 per cent  $\alpha$ -methylglucoside, and 1 cc. of buffer of pH 4.4, 5.5, and 7.0 (0.1 N acetate), respectively, were added. There was no hydrolysis after 3 days at 30°.

*Optimum pH*—As shown in Table I the most favorable pH for the activity of the maltase from the mammary gland lies between pH 6.6 and 7.4, with a maximum activity at pH 6.8 to 7.0. For this work the purified extract only was used.

#### *Glucomaltase, $\alpha$ -Glucosidase, and Specificity of Invertases*

In 1925, Leibowitz (Leibowitz and Mechliniski (10) and Leibowitz (11)) proposed a theory of the existence of two kinds of maltase. One kind, which is present in yeast and has the power to hydrolyze both maltose and  $\alpha$ -methylglucoside, and another

kind which is found in taka-diastrase, *Aspergillus wentii*, and germinated barley (12, 13) hydrolyzes only maltose. Thus he would differentiate between glucosidomaltase and glucomaltase. Fischer and Niebel (14) had found as early as 1896 that the maltase which they obtained from horse and beef serum would only hydrolyze maltose and not  $\alpha$ -methylglucoside and in 1898 Fischer (15) had found that yeast hydrolyzes both  $\alpha$ -methylglucoside and maltose. Weidenhagen (16), who has studied yeast maltase, denies the existence of a special sucrase, maltase, or  $\alpha$ -glucosidase. He assumes that sucrose ( $\alpha$ -glucosido- $\beta$ -*h*-fructoside) is hydrolyzed by either an  $\alpha$ -glucosidase or a  $\beta$ -*h*-fructosidase, and maltose ( $\alpha$ -glucosidoglucose) only by  $\alpha$ -glucosidase, and according to his steric configurative theory of the specificity of invertases, maltase wherever found in nature must hydrolyze sucrose, which contains  $\alpha$ -glucose as a constituent, and must also split  $\alpha$ -methylglucoside. Weidenhagen's work (17) has been seriously questioned by Karstroem (18), Myrbaeck (19), Virtanen (20), and by Pringsheim, Borchardt, and Loew (21).

#### DISCUSSION

In Experiment I it has been shown that there are no proteases in the mammary gland and therefore autolysis which occurs in such extracts is not necessarily due to the action of proteolytic enzymes as assumed by Grimmer, whose only experimental proof was that some of the tissue went into solution on standing at room temperature. Experiments III and IV show that the hydrolysis of salol and of starch by extracts of mammary tissue observed by Grimmer was due only to the hydrolytic action at the hydrogen ion concentration of the extract. In confirmation of the work of Bradley, we have been unable to demonstrate, by modern methods, the presence of lactase, or of more than a trace of lipase in mammary tissue, nor is there a lactose-synthesizing enzyme (Experiment V). A maltase similar in its properties to that which Leibowitz obtained from malt and to that of another maltase obtained from *Bacillus coli* by Karstroem, has been found by us in the mammary gland (Experiment VII). The maltase is inactivated by heat. It hydrolyzes maltose with fair rapidity (Table I) but it is inert toward  $\alpha$ -methylglucoside and toward sucrose. Hitherto maltase has not been found in the mammary

gland. The question as to whether maltases of varied origin are identical has been much debated. Such an invertase might be responsible for the presence of lactose in milk, polymerizing maltose from glucose, and by a special stereokinetic property it might further convert maltose into lactose. Since our extracts did not possess these properties, nor the power of converting sucrose into lactose we cannot subscribe to the theory of Roehman and of Hesse, that stereokinases of this nature are present in the gland. As shown in Table I, the maltase of the mammary gland is most active at about the neutral point (6.8 to 7.0) which is very close to the optimum pH of yeast maltase found by Willstätter and Baman (22). The optimum pH of taka-maltase differs from

TABLE I  
*Optimum pH for Activity of Maltase of Mammary Gland*

pH	Per cent hydrolysis	pH	Per cent hydrolysis
5.4	28	6.8	60
5.8	35	7.0	58
6.2	40	7.4	56
6.6	54	7.6	52

To 2 cc. of the dialyzed maltase (watery suspension of the precipitate which formed on dialysis) 1 cc. of 0.1 N acetate buffer and 1 cc. of 1.42 per cent maltose were added. The time of incubation was 4 hours and the temperature was 30°.

that of the other maltases mentioned; it is at 2.5 to 8.0 (10). Although our preparation is most active at about the same range of pH as is the optimum for yeast maltase it cannot be the same enzyme since it does not split  $\alpha$ -methylglucoside. Our stand in this controversy, based on our experiments, is that there are distinct glucomaltases, which hydrolyze maltose but not sucrose nor  $\alpha$ -methylglucoside. The maltase of mammary tissue is an example.

#### SUMMARY

1. The mammary gland has been tested for a series of enzymes of which only maltase was found to be present.
2. The absence of lactase and of an appreciable amount of lipase corroborates earlier investigators, who sought the mech-

anism of the synthesis of lactose and fat by reversible reactions of these enzymes.

3. Properties of the newly found maltase have been studied and compared with those from other sources. The theory of Leibowitz of the existence of distinct glucomaltases as distinguished from glucosidomaltases has been corroborated.

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# A METHOD FOR THE DETERMINATION OF MONOSACCHARIDES IN THE PRESENCE OF DISACCHARIDES AND ITS APPLICATION TO BLOOD ANALYSIS\*

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While attempting to determine the presence of lactase in mammary tissue, it became evident that no satisfactory method for the determination of the hydrolytic products of lactose in the presence of the substrate was available. We, therefore, sought to modify the Barfoed test in order to make it practical for quantitative, as well as qualitative, procedures.

Since the publication of Barfoed's test many other attempts have been made to improve it. Barfoed's reagent consists of copper acetate and acetic acid. The modifications vary as to the proportions of these two constituents used and as to the time of boiling (1). Too high acidity and prolonged boiling both result in disaccharides giving positive tests (2, 3). The difficulties which arose were due chiefly to the volatilization of the acetic acid. Sieben (4), in order to avoid the loss of the acetic acid, used sealed containers and heated the fluid at 40°. Legrand (5), employing Barfoed's reagent in volumetric work, collected the  $\text{Cu}_2\text{O}$  which formed on oxidation of the sugars, on filter paper, treated it with  $\text{Fe}_2(\text{SO}_4)_3$ , and then titrated with  $\text{KMnO}_4$ . This and the interesting study of Hinkle and Sherman (6) gave promise of the usefulness of this reagent for the colorimetric determination of monosaccharides in the presence of disaccharides.

The principle of the new method is the same as of those devised by Folin and Wu and by Benedict, for the determination of reducing sugars, the only difference being that the solution containing the

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sugars is heated with a non-volatile acid copper solution instead of an alkaline one. The cuprous oxide which forms on reduction is treated as in the methods mentioned, with an acid molybdate solution and the blue color obtained compared with a standard.

We found that the ordinary Barfoed's reagent (copper acetate and acetic acid) when boiled with glucose solutions gave amounts of cuprous oxide not proportional to the quantity of sugar used. This was found to be due to the volatilization of the acetic acid and the consequent formation of basic salts. We replaced the acetic acid with lactic acid, and found that the cuprous oxide formed was proportional to the amount of monosaccharide used. The reagent as modified in this manner did not give a positive test with fairly large amounts of disaccharides even after much longer boiling than was usual in earlier modifications.

This new colorimetric method is also useful for the study of saccharases and glucosides. Of a large number of glucosides, which we tested, nearly all gave reductions with alkaline copper solutions but none with the lactic acid reagent. The usefulness of this method would seem to be very wide. Phlorhizin was long considered to be an  $\alpha$ -glucoside and only recently (7) was it found to be hydrolyzed by emulsin. This was due to the very slow velocity at which its splitting takes place. Phlorhizin seems to be one of the most slowly hydrolyzed  $\beta$ -glucosides. By our method the effect of emulsin on phlorhizin may be detected within a few hours. Polarimetric methods, which have been used to determine glucoside hydrolysis in most investigations, present certain difficulties. The method about to be described is simple, rapid, and requires very little material. It may be used qualitatively as well as quantitatively.

### *Solutions*

*New Acid Copper Monose Reagent*—Dissolve 24 gm. of copper acetate (Merck, normal, c.p.) in 450 cc. of boiling water. If a precipitate forms do not filter. Add immediately 25 cc. of 8.5 per cent lactic acid (Mallinckrodt, United States Pharmacopœia X, 85 per cent) to the hot solution. Shake; nearly all the precipitate will dissolve. Cool, dilute to 500 cc., and after sedimentation filter off the impurities.

*Standard Monose Solutions*—Two standard monose solutions

are needed, one containing 0.15 mg. of monose per cc. and one containing 0.3 mg. of monose per cc. Toluene serves as a good antiseptic and does not interfere with the test.

*Color Reagent*—This reagent is the same as described recently by Benedict (8). It was found to be extremely useful for this method. For convenience we give the preparation of the reagent. Place 150 gm. of pure molybdic acid (we used Eimer and Amend's "free of ammonia") in an Erlenmeyer flask and add 75 gm. of pure anhydrous sodium carbonate. Add water in small portions, with shaking (about 500 cc.). Heat to boiling or until all of the molybdic acid has been dissolved. Filter off the insoluble matter. At this point we noticed only a small trace of insoluble substances. We therefore omitted the washing of the precipitate with hot water as suggested by Benedict. Add 300 cc. of 85 per cent phosphoric acid to the filtrate, cool, and dilute to 1 liter.

When fresh, and even after 4 months, the new copper monose reagent gives no color after 8 minutes boiling with 2 mg. of lactose and the addition of the color reagent and a very slight color with 3 mg. It gives a very good color with 0.1 mg. of glucose, the intensity of which is the same whether lactose is present or not (see Table I). The smallest amount of the various monoses which can be accurately determined is 0.1 mg. With pure bioses the minimum amount which gives reduction should be determined whenever the monose reagent is freshly prepared.

*Tubes*—Since there is no reoxidation in this method, any tubes marked at 25 cc. may be used. Benedict's sugar tubes serve the purpose.

*Determination*—Place in a tube 2 cc. of the *neutral* monose-biose solution (or the solution to be tested)<sup>1</sup> having a *total* reducing value<sup>2</sup> of not more than the equivalent of 2.5 mg. of glucose and not less than the equivalent of 0.1 mg. of glucose, in the case of glucose-lactose mixtures; or not more than the equivalent of 1 mg. of glucose in glucose-maltose mixtures. With sucrose, even 5 mg. of this sugar do not interfere. Transfer to two separate

<sup>1</sup> The solution should contain a minimum of inorganic salts; *e.g.*, more than 3 mg. of NaCl per 2 cc. of the solution to be tested interfere (see also Welker (9)). The pH should be as close to 7.0 as possible.

<sup>2</sup> The total reducing value may be determined by any method, such as the Folin-Wu or Benedict (8) method.

tubes 2 cc. of each of the two standards. Add 2 cc. of the monose reagent to each of the three tubes. Heat in boiling water for 8 minutes (time is an important factor). Cool for 2 minutes. Add 2 cc. of the color reagent. Mix the contents and after 2 minutes further standing add water to the 25 cc. mark in the case of the standards and dilute the unknown to a suitable mark. Mix thoroughly and compare with the standard colorimetrically.

$$\text{Calculation—} \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.3 \text{ or } 0.6 \text{ (i.e., mg. monose in standard)} \times \frac{\text{volume of unknown}}{\text{volume of standard}} \text{ equals mg. monose in unknown.}$$

TABLE I  
*Mixtures of Glucose\* and Lactose†*

The total volume of all samples is 2 cc.

Experiment No.	Glucose	Lactose	Glucose found by new method	Experiment No.	Glucose	Lactose	Glucose found by new method
	mg.	mg.	mg.		mg.	mg.	mg.
1	0.10	0.90	0.10	8	0.80	0.20	0.81
2	0.20	0.80	0.20	9	0.90	0.10	0.92
3	0.30	0.70	0.30	10	1.00	0.00	1.02
4	0.40	0.60	0.39	11	0.00	2.00	0.00
5	0.50	0.50	0.51	12	0.20	2.50	0.21
6	0.60	0.40	0.60	13	0.00	3.00	Very slight trace
7	0.70	0.30	0.72				

\* Merck, C.P.; other pure preparations gave similar results.

† Mallinckrodt, C.P.; other pure preparations gave similar results.

In Table I is shown the result of glucose determination in glucose-lactose mixtures. It will be seen that 2.5 mg. of lactose are the maximum amount which may still be present in the mixture without causing interference. In Table II, Experiment 6 shows that 2 mg. of maltose interfere and in Table III it is shown that as much as 50 times as much sucrose as fructose allows the exact determination of the monose present. In all three sets of experiments the determination of a monose in the presence of a biose was accomplished with reasonable accuracy, great ease, and rapidity.

*Application to Determination of Monose in Blood*

Since this method yields excellent results when employed for the determination of monoses in aqueous solutions, it seemed desirable to attempt to apply it to blood sugar determination.

TABLE II  
*Mixtures of Glucose and Maltose\**

The total volume of all samples is 2 cc.

Experiment No.	Glucose	Maltose	Glucose found by new method
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	0.10	0.90	0.11
2	0.20	0.80	0.20
3	0.40	0.60	0.41
4	0.80	0.20	0.80
5	1.00	0.00	0.98
6	0.00	2.00	Slight trace

\* Kahlbaum; other pure preparations gave similar results.

TABLE III  
*Mixtures of Fructose\* and Sucrose†*

The total volume was 2 cc. in all samples.

Experiment No.	Fructose	Sucrose	Fructose found by new method
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	0.10	5.00	0.10
2	0.20	5.00	0.21
3	0.40	5.00	0.39
4	0.80	5.00	0.82
5	1.00	5.00	1.03
6	0.00	1.00	0.00
7	0.00	5.00	0.00

\* Kahlbaum; other pure preparations gave similar results.

† Commercial.

For this purpose it is only necessary to have a neutral filtrate with a minimum of electrolytes. Such a filtrate is obtained by the iron precipitation method of Steiner, Urban, and West (10). This yields automatically a filtrate having a neutral pH, with no

increase in electrolytes, and is claimed to yield only true sugar values. By using this precipitation procedure and our monose method, results were obtained which appeared to be very satisfactory.

#### *Qualitative Test for Monosaccharides*

*Method A*—Determine the total reducing value of the solution of reducing sugars to be tested by the volumetric sugar method of Benedict (11), or by any other quantitative method. Dilute so as to contain 0.1 per cent of total reducing sugars (mono- and disaccharides). If the sugar mixture is in dry form make a 0.1 per cent solution. Transfer to a test-tube 1 cc. of the 0.1 per cent solution. Place in a separate tube 1 cc. of distilled water, to serve as a control. Add 1 cc. of the new acid copper reagent to each of the two tubes. Heat in boiling water for 3 minutes. Cool for 2 minutes. Add 1 cc. of the color reagent to each. Mix. A blue color will be obtained if a monose is present; but the solution will have the same color as the control if only bioses are present.

Chlorides interfere with the test (9), but we found up to 5 mg. of NaCl in 1 cc. of the 0.1 per cent sugar solution to be without effect.

Under these conditions exact results may be obtained easily by this new method. The smallest amount of glucose (or other monosaccharide) which can be detected in the presence of a disaccharide is 0.1 mg. in 1 cc. of the solution. There may be 10 times this amount of maltose, or 25 times this amount of lactose present in the monose-biose mixture, without causing interference.

*Method B*—A more rapid but less exact procedure may be performed in the following manner. Transfer to a test-tube 1 cc. of the solution to be tested. Add 1 cc. of the new acid copper reagent. Heat in boiling water for 3 minutes. A red precipitate ( $\text{Cu}_2\text{O}$ ) will indicate the presence of 0.1 per cent or more of monose if not more than 4 per cent of lactose, 2 per cent of maltose, or 10 per cent of sucrose is present.

#### SUMMARY

1. A new colorimetric method for the determination of monoses in the presence of reducing bioses has been described. It is rapid,

accurate, and simple. The method is also useful in the study of saccharases and glucosidases respectively.

2. The method has been applied to the determination of sugar in suitable blood filtrates.

3. An exact qualitative method for the differentiation of monosaccharides and disaccharides has also been given.

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# THE CHEMICAL NATURE OF EMULSIN, RENNIN, AND PEPSIN\*

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## INTRODUCTION

Several years ago the long current belief that enzymes were proteins was suddenly subjected to searching criticism. A number of papers appeared describing protein-free enzymes. Many workers stated that their enzymes contained almost no protein, thus emphasizing the purity of their preparations. Contrary to these findings considerable work has been done recently to indicate that the enzymes, urease (1-5), pepsin (6), trypsin (7), amylase (8), and rennin (9), are of protein nature. A recent controversy on the chemical nature of urease between Waldschmidt-Leitz and Steigerwaldt (10) and Sumner and Kirk (11), seems to be settled in favor of the latter workers, that crystalline urease is a protein. Although Sherman and his associates (12) could not obtain a protein-free amylase preparation by the adsorption method of Willstätter, Waldschmidt-Leitz, and Hesse, Waldschmidt-Leitz and Reichel (13) state that they were able to prepare an active protein-free amylase. In a recent paper Willstätter and Rohdewald (14) confirmed the earlier (1885) finding of Sundberg (15) of the preparation of a protein-free pepsin solution. These authors followed exactly the method described by Sundberg; *i.e.*, extraction of the mucosa of the calf stomach with saturated NaCl solution for several days and autodigestion for 2 weeks, followed by precipitation with calcium chloride, sodium phosphate, and ammonia, and solution of the resulting precipitate by means of HCl and dialysis. They state that after dialysis of

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the final solution there was an unexpected loss of active material, yielding only a very slightly active solution, but that *this solution did not give any of the protein color tests*. Willstätter and Rohdewald also quote the work of Bruecke (16) who had as early as 1861 obtained protein-free pepsin "*solutions*," by adsorption. Concerning Northrop's crystalline pepsin Willstätter and Rohdewald state: "Natürlich sind die Eigenschaften des von J. H. Northrop in der Form von krystallisiertem Eiweiss erhaltenen Pepsin ganz andere. Diese gibt z. B. stark positive Xanthoproteinreaktion. Es enthält auch viel vom Träger der Millionreaktion, es liefert nämlich nach Northrop bei der Selbstverdauung eine grosse Menge von Tyrosinkrystallen." On p. 261 of the paper of Willstätter and Rohdewald we read the following quotation from a letter of Northrop regarding the protein nature of pepsin: "The proteolytic properties and the protein properties are both attributes of the same molecule."

We have subjected the question of the protein nature of several enzymes to study from two different angles. We will show that most of the protein of an emulsin preparation can be digested with proteases and that the solution when dialyzed will yield negative protein color tests but that after concentration of the dialyzed solutions in a vacuum the color tests become positive. In the earlier reports and in that of Willstätter and Rohdewald only the solutions which were subjected to considerable digestion and dialysis were tested for protein. In the paper of Willstätter and Rohdewald these dialyzed solutions of very slight activity were called preparations yielding negative protein color tests. It is obvious that although most of the protein can be removed (rennin dialyses through membranes (9, 17)) by the method of Sundberg, most of the enzyme is likewise lost and such a solution having only very slight enzyme activity will at most contain only traces of protein, which very readily escape detection. We will also show that crystalline pepsin and highly purified rennin, although undoubtedly containing protein, show no protein color tests when diluted as high as the so called protein-free enzyme preparations of Willstätter and Rohdewald.

Emulsin was claimed by Oka (18) and recently by Nordefeldt (19) to be of non-protein nature. More recently, however, Helferich and Bredereck (20) obtained preparations having con-

siderably higher activity and state that these still contain protein. The preparation of Okta was obtained by tryptic digestion, dialysis, and subsequent precipitation with alcohol, that of Nordefeldt by lead acetate precipitation, decomposition of the lead compound by  $H_2S$ , and dialysis. In this work we undertook to prepare protein-free emulsin by the methods of Okta, and of Nordefeldt, and by other procedures. Because of the statement of Oppenheimer (21) that pepsin inactivates emulsin, we have repeated this experiment as well, since it obviously bears on the question of the chemical nature of emulsin.

#### EXPERIMENTAL

*Chemical Nature of Emulsin*—The method for the preparation of the emulsin, which we used for the experiments in Table I and which we also used as starting material of "protein-free" emulsin according to Okta's directions, is as follows:

*Preparation of Emulsin*—300 gm. of sweet almonds were suspended in water at room temperature for 20 minutes. Then the skin was removed mechanically and the almonds after being dried in the air were ground to a fine meal and some of the oil pressed out. The remainder of the oil was extracted with three 300 cc. portions of ether. It was now strained through cheese-cloth. The weight of oil-free meal was found to be 110 gm. It was now extracted with 800 cc. of 33 per cent ethyl alcohol by stirring with a glass rod for 6 minutes, after which it was filtered through filter paper. 500 cc. of filtrate were obtained. To this, 500 cc. of 95 per cent ethyl alcohol were added. The precipitate which formed was centrifuged off and dried over sulfuric acid at room temperature. The yield was 2.5 gm. of dry emulsin, having an activity about 10 times that of the original 33 per cent alcoholic extract, when salicin or lactose was used as a substrate. To aid in solution of the dry emulsin a few drops of NaOH solution were added to the watery suspension and immediately neutralized to pH 7.

*Activity Determination*—To 3.2 cc. of enzyme solution 1 cc. of a 1.42 per cent of lactose solution (or 2.5 per cent salicin solution) and 0.8 cc. of 0.1 N acetate buffer of pH 4.4 were added and incubated at 30°. At appropriate times 1 cc. of the digest was transferred to another vessel, 0.5 cc. of N NaOH was added to stop

TABLE I

*Digestion of Emulsin by Proteolytic Enzymes*

Performed at 37°.

Experiment No.	Proteolytic enzyme	pH	Per cent inactivation as compared to buffered, boiled control		
			Original	Control	Time of incubation
1	Pepsin, Park, Davis and Co., U.S.P.	4.4, 0.1 N acetate buffer	No inactivation*	No inactivation*	2 wks.
2	" "	Adjusted to pH 2.0 with HCl	100	100	3 days
3	Trypsin, Fairchild, U.S.P.	7.0, 0.1 N acetate buffer	No inactivation	No inactivation	2 wks.
4	" "	Adjusted to pH 8 with NH <sub>3</sub> ; more NH <sub>3</sub> added as needed	" " *	" " *	2 "
5	After 2 wks. peptic digestion as in Experiment 1, trypsin added and adjusted to pH 8.0	" "	" " *	" " *	2 "
6	Pancreatin, Coleman and Bell	" "	" " *	" " *	2 "

\* In these experiments slight inactivation took place due to buffer action.

The proportions of the digestion mixtures in Experiments 1 to 6 were: To 10 cc. of 1 per cent emulsin solution 3 cc. of a 1 per cent protease and 1.6 cc. of acetate buffer were added (unless otherwise stated). Of the pancreatin 0.2 gm. was dissolved in 50 cc. of water and added to 50 cc. of a 2 per cent solution of the concentrated emulsin. Experiments 4 and 6 were also performed on a larger scale; 10 gm. of emulsin were suspended in 1 liter of distilled water, 1 gm. of pancreatin was added and adjusted with NH<sub>3</sub> to pH 8.0. After 4 weeks digestion it was dialyzed for 3 weeks after which the solution gave negative color tests but when concentrated in a vacuum at room temperature and precipitated with alcohol they all gave positive biuret and weak xanthoproteic tests. The activity of these emulsin preparations was very slight and the loss of active material was enormous.

The results as to inactivation with proteases were similar when gum was added to the various samples (5).

In all experiments toluene was used as an antiseptic.

the enzyme action, and after 10 minutes 0.5 cc. of *N* acetic acid. The reducing power was then determined by the monose method of Tauber and Kleiner (22).

*Preparation of "Protein-Free" Emulsin by Digestion Method of Okta*

Experiments 3 to 6 of Table I repeat the work of Okta; *i.e.*, tryptic digestion at an alkaline pH, filtration, subsequent dialysis (we found that emulsin tends to dialyze through collodion bags), and then precipitation with alcohol. The method of Okta was repeated several times and since we could not obtain protein-free emulsin by this method, as shown in Table I, we tried to remove the proteins after digestion and dialysis by salting out with saturated solutions of neutral salts, or by fractional precipitation with alcohol or acetone at various pH's. No separation of the protein from the active part could be obtained by any of these methods. The protein precipitate included the active emulsin and the supernatant fluids were found to be inactive.

*Preparation of "Protein-Free" Emulsin by Lead Acetate Method of Nordefeldt*

Although Nordefeldt's directions were carefully followed, the vacuum-dried sample still gave a strong biuret test. The lead precipitation was repeated, but this also gave yields containing protein. The N content of Nordefeldt's preparation was 12.3 per cent and still contained carbohydrates according to this author. We also found that emulsin obtained by this method contained much pentosan besides proteins.

*Experiments to Show That Dilute Enzyme Solutions May Apparently Contain No Protein*

*Protein Color Tests on Purified Rennin*

*Biuret Test*—100 mg. of the purified rennin<sup>1</sup> were dissolved in 100 cc. of 0.1 *N* HCl. 1 cc. of the solution containing 1 mg. of

<sup>1</sup> The purified rennin was prepared according to the method of Tauber and Kleiner (9) and had a milk-clotting activity of 1:4,850,000 as determined by their method (9). It had no peptic activity. The crystalline pepsin (kindly furnished by Dr. J. H. Northrop) had a milk-clotting activity of 1:800,000 and a peptic activity of 14 cc. of 0.1 *N* NaOH per gm. as determined by the formol method (6).

rennin gave a slightly pink biuret test. Then the solution was diluted 10 times. 1 cc. of this diluted solution containing 0.1 mg. of rennin, capable of clotting 485 cc. of milk in 10 minutes at 40°, gave a negative biuret test.

*Xanthoproteic Test*—To 1 cc. of the rennin solution containing 1 mg. of rennin, 1 cc. of concentrated  $\text{HNO}_3$  was added. A slightly yellow color was obtained. The 10 times diluted rennin solution gave a negative xanthoproteic test.

#### *Protein Color Tests on Crystalline Pepsin*

*Biuret Test*—100 mg. of crystalline pepsin<sup>1</sup> were dissolved in 100 cc. of 0.1 N HCl. 1 cc. of this solution gave a slightly violet biuret test. The pepsin solutions diluted 10 times so as to contain 0.1 mg. of pepsin per cc. and power to clot 80 cc. of milk gave a negative biuret test.

*Xanthoproteic Test*—The test performed in exactly the same manner as for rennin gave similar results.

#### DISCUSSION

Proteases do not digest emulsin (Table I), which is contrary to Oppenheimer's findings. Acid (pH 2), however, inactivates the enzyme rapidly (see control of Experiment 2). When gum was added (5) the results were similar; *i.e.*, no inactivation of the emulsin by the proteases took place. However, we observed that most of the protein of the emulsin preparation had been digested during 2 to 4 weeks of incubation at 37° without decreasing enzyme activity *per se*. Repetition of the work of Okta, and that of Nordefeldt did not yield a non-protein emulsin; nor did various other methods. It is interesting to note that neither Okta nor Nordefeldt accounted for the proteins of the proteases they used. It was found by Northrop (6) that pepsin, although a protein, does not digest itself, but when in solution, it becomes gradually inactive and the inactive part is digested by the active one. Thus we may suppose that antitryptic and antipectic groups protect active enzymes, in the intestine for instance, from being digested by one another or by themselves.

In the above color tests it has been shown that rennin and pepsin solutions which were diluted did not respond to protein color tests, although their activity is certainly not less than that

of the enzyme solutions of Willstätter and Rohdewald. Our diluted rennin solution which did not give protein color tests was still quite active since 1 cc. containing 0.1 mg. of rennin would clot 485 cc. of milk in 10 minutes at 40°; and 1 cc. of crystalline pepsin solution containing 0.1 mg. of pepsin per cc. coagulated 80 cc. under the same conditions.

## SUMMARY

1. Emulsin is not inactivated by pepsin, trypsin, or pancreatin.
2. Acid (pH 2) inactivates emulsin rapidly.
3. The preparation of a protein-free emulsin by methods of other investigators could not be reproduced. Reasons are advanced for believing that their preparations were not protein-free. Various other procedures of freeing the enzyme from protein were tested but invariably the proteins were not removed.
4. A practical method for the preparation of emulsin is given.
5. It has been demonstrated that dilute active enzyme solutions may easily escape the protein color tests, thus leading to the erroneous interpretation that enzymes are non-protein substances.

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## IRON AND COPPER IN LIVER AND LIVER EXTRACTS

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The anemia problem is actually approached from two different sides. On one side, we find the liver treatment as introduced by Minot and Murphy and supplemented by a large number of other authors. On the other side, the publications of Elvehjem, Hart, and their coworkers showed the importance of the combined action of iron and copper. The fact that liver has been used especially in primary anemias, whereas iron and copper seem to be effective more in secondary anemias, probably explains why so few attempts have been made to connect both methods of research.

It can be considered as an established fact that the fraction of liver extract effective in pernicious anemia, as prepared by Cohn *et al.* (1), has little value in secondary anemia. On the other hand, Whipple, Robscheit-Robbins, and Walden (2) describe a liver fraction potent in hemorrhagic anemia, *i.e.* a secondary type of anemia. A recent publication by Schultze (3) expresses the opinion that the value of liver in anemia treatment is largely due to its copper content and that copper stimulates the formation of red blood cells, while iron stimulates hemoglobin formation. The latter assertion seems extremely doubtful in view of the work of Elvehjem *et al.*, which proved that hemoglobin cannot be formed in the absence of copper, regardless of the amount of iron given (4).

The following work was done to show the distribution of both metals in different mammalian livers and the accumulation in certain fractions of their extracts. The methods of fractionating the extracts excluded from the beginning a quantitative separation of the metals, so that none could be completely absent in any

fraction. The minimum of copper required to permit hemoglobin formation has not yet been determined. It might be possible that beyond a certain optimum, which probably is very low, increased copper doses will not be of any benefit. The conclusion that a high copper content has a special antianemic value is not possible; it might be with reference to iron, but there also, the evidence must be established by experimental physiological and clinical experience.

For the iron determination, the method described by Elvehjem and Peterson (5) has been used. The Biazzo method, modified by Elvehjem and Lindow (6), was used for the copper determination. In most of our determinations, the copper had to be separated by means of hydrogen sulfide on account of the disturbing presence of iron. From the tables given by Elvehjem *et al.* (5-7) the following data are of interest for this investigation.

Dry beef liver.....	0 0294	per cent Fe
Fresh " " .....	0 0083	" " "
" hog " (with 31.3 per cent dry matter).....	0 0391	" " "
Dry beef liver.....	71	mg. Cu per kilo
	75.7	" " " "
" hog " .....	20 8	" " " "

The analysis of a commercial liver extract (360 mg. of Cu per kilo) is of little significance, since one knows neither the kind of liver used, nor the fraction of liver extract represented. One finds, too, that during the process of manufacture, commercial liver extract comes in contact with copper. Some time ago, Dr. Elvehjem had the kindness to analyze a sample of dried horse liver which was submitted by one of us. He found 30 mg. of Cu per kilo.

As the liver is an organ of storage of many substances, it might be expected that the content of metals would be subjected to the same degree of variation as, for example, that of glycogen or fat. Determinations which we made previously showed that this is true to a certain extent; however, the comparison of livers from different animals showed that those from some species are always higher in Cu or Fe than others.

#### *Preparation of Material*

The extracts and fractions for the determination were prepared in glass, thus avoiding any possible contamination with metals.

A part of the ground liver was dried at 105–110° and the moisture content determined. The dry product was used for the Cu and Fe analysis. A larger quantity of finely ground liver, usually 1 kilo, was mixed with the same quantity of water and left for 5 hours, with constant stirring, at room temperature. Considering the practical impossibility of a 100 per cent extraction, a complete exhaustion of the liver was not attempted. The large quantities of liquid, which an approximately perfect extraction would give, would make it impossible to handle and evaporate the whole batch in glass. It was supposed that after 5 hours an equilibrium of the diffusible substances would be established inside and outside of the cells. With consideration of the dry matter present in the fresh liver and in the residue, of the quantities of both, and the quantities of liquid obtained, an extraction of about 60 per cent was calculated. This calculation is based on the assumption that an equilibrium of the fluid is obtained, which may be subject to criticism, but so would be any other supposedly complete extraction. The mixture was heated to about 85° and pressed out. The weight of the pressed residue and its moisture content were determined. In some determinations, a part of the water extract was evaporated to dryness, the residue calculated for 1 kilo of liver, and analyzed. The larger part of the extract, and in other experiments the whole extract, was evaporated *in vacuo* to 65 cc., and sufficient 95 per cent alcohol was added to obtain a final concentration of 67 per cent alcohol. The precipitate obtained, which would represent Whipple's fraction active in secondary anemia (2), was dried, weighed, and analyzed. We have called this product Fraction S. The alcoholic liquid, which contains the factor active in pernicious anemia, was brought to dryness in some batches, then weighed, and analyzed. This raw product is Fraction PA. In other batches this product was fractionated. The alcoholic liquid was evaporated *in vacuo* to a volume of 50 cc. and 10 cc. of alcohol again added. On standing, a precipitate, mostly of small volume, fell out (Fraction F). It was practically absent in beef and hog liver. The filtered liquid was stirred in such a quantity of absolute alcohol that a concentration of more than 93 per cent was obtained. The precipitate, which corresponds closely to Cohn's Fraction G, is kept with that designation. By evaporating the alcoholic liquids, a dark hygroscopic end fraction was obtained, Fraction E.

TABLE I  
*Fe and Cu in Liver and Liver Extracts*

Sample No and kind of liver	Fraction derived from it	Quantity from 1 kilo fresh liver	Dry matter	Fe in		Cu in	
				Dry matter	Whole fraction	Dry matter	Whole fraction
		<i>gm</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg per kg</i>	<i>mg</i>
1. Horse	Whole liver		28 2	0 075	209	27 3	7 70
	Pressed residue	760	32 8	0 055	137	16	4 0
	Whole H <sub>2</sub> O extract	32	100	0 22	70	104	3 3
	S	14 4	100	0 51	73	170	2 44
	PA. Solution in 67% alcohol	17 7	100	0 007	1 2	40	0 70
2. "	Whole liver		28 3	0 090	255	25	7 07
	Pressed residue	852	29 5	0 078	196	16	4 0
	Whole H <sub>2</sub> O extract	30	100	0 22	66	88	2 64
	S	14	100	0 50	70	172	2 40
	PA. Solution in 67% alcohol	18	100	0 0033	0 6	34	0 61
3. "	Whole liver		28 5	0 085	242	28	8 0
	Pressed residue	720	35 7	0 055	142	21	5 4
	S	10 7	100	0 98	105	181	1 94
	F	1 92	100	0 01	0 2	55	0 11
	G	5 72	100	0 033	1 9	50	0 29
	E. Solution in 93% alcohol	7 5	100	0 0045	0 33	19	0 14
4. Dog	Whole liver		25 0	0 10	250	44	11
	Pressed residue	712	28 0	0 072	144	20	4 0
	S	37	100	0 32	118	185	6 8
	F	1 17	100	0 0048	0 056	300	0 35
	G	4 5	100	0 0045	0 20	54	0 24
	E. Solution in 93% alcohol	18	100	0 0		11	0 20
5. Beef	Whole liver		28 0	0 022	61 6	57	16
	Pressed residue	800	30 7	0 020	48 6	47	11 5
	Whole H <sub>2</sub> O extract	33	100	0 036	11 9	120	3 96
	S	13 8	100	0 07	10	260	3 6
	PA. Solution in 67% alcohol	19	100	0 0035	0 66	6	0 11
	G	7 5	100	0 01	0 75	13	0 1
	E. Solution in 93% alcohol	11	100	0 0	Trace	Tr	Tr.
6. Hog	Whole liver		29 7	0 09	267	16	4 75
	Pressed residue	900	31 0	0 073	203 7	13	3 63
	S	8 6	100	0 75	64 6	90	0 78
	G	4 42	100	0 058	2 6	29	0 13
	E. Solution in 93% alcohol	5 78	100	0 0		14	0 09

In Sample 5 the findings for Fraction F, medium fraction, were insignificant; in Sample 6, the results were negative.

The analytical results are presented in Table I. It can be seen from these findings that the metals are extracted to a rather incomplete degree. Even if we consider that 40 per cent of the soluble metal was left in the residue by incomplete extraction, hardly more than half of the iron and copper could be considered as water-soluble. But the percentage of soluble metal is higher than the percentage of total soluble matter. The dried aqueous extract shows a considerable increase in Cu and Fe. The precipitate obtained with 67 per cent alcohol carries almost all of the iron and the largest part of the copper. The iron content in this fraction has values between 0.5 and 1 per cent in horse or hog liver, whereas in beef liver it was rather low, due to the small iron content in beef liver itself. In copper, the beef liver fraction leads with 260 mg., horse and dog liver have values of 170 to 180 mg., and hog liver 90 mg. This fraction may or may not contain a specific principle active in secondary anemia, but the metal content alone would explain the results reported by Whipple *et al.* (2). The portion of metals still in solution in 67 per cent alcohol comes out with the following precipitations. The end fraction (F) contains practically no iron at all. The copper decreases in each fraction, with exception of the medium fraction (F) in dog liver. The most interesting part is Fraction G, on account of its use in pernicious anemia. The copper content in this fraction is only slightly higher than in the liver itself, and in the case of beef liver even lower. On the other hand, the therapeutic value of Fraction PA as well as of Fraction G has been proved beyond any doubt (8, 9). It is difficult to believe that the copper is the stimulating agent for the formation of erythrocytes, as supposed by Schultze (3). In that case, Fraction S should be the more valuable part of the liver. It can be concluded from these results that the substance active in pernicious anemia has no relation to the copper and iron content of the liver, whereas the value of liver in secondary anemia may be at least partly due to the presence of these metals.

#### SUMMARY

In the livers investigated, horse, hog, and dog livers are uniformly high in iron, whereas beef liver contains considerably less. The copper is highest in beef liver, less in dog, horse, and hog liver.

Only a part of these metals can be extracted with water. They are accumulated in the fraction which is precipitated with 67 per cent alcohol. The fraction obtained afterwards with higher grade alcohol (up to 93 per cent) contains less copper and very little iron.

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## THE SOLUBILITY OF CALCIUM STEARATE IN SOLUTIONS CONTAINING BILE AND IN WATER

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The principal functions of bile in the process of digestion are (a) to emulsify fats, by lowering the surface tension of the intestinal contents, (b) to bring fats, fatty acids, and soaps into true solution, and thus to make them diffusible, and (c) to aid in the control of the reaction of the intestinal contents, by virtue of its high concentration of bicarbonate ion. The other functions are mainly excretory. That bile increases the solubility of substances such as fatty acids and soaps is of interest in relation to the maintenance of the calcium level of the blood. It is thought that bile renders calcium soaps soluble, and that the dissolved calcium soaps diffuse readily into the blood. To support this idea, one finds in the literature data which have led to the following conclusions.

In the absence of bile from the intestine, large amounts of fatty acids and soaps are eliminated in the feces from fat-containing diets (1). Clear, diffusible solutions of fatty acids with the salts of taurocholic and glycocholic acids have been prepared (2). The removal of bile by fistula prevents a rise of blood calcium which occurs normally after the ingestion of milk; addition of bile salts to the milk brings about a rise of blood calcium (3). Bile salts increase the solubility of calcium soaps (4). After administration of sodium cholate to dogs, the calcium of the bile is increased (5), and the elimination of calcium in the feces is lessened by the administration of cholic acid (6). Cholic acid produces a hypercalcemia (7). Calcium soaps seem to be absorbed without dissociation (8). In the changing of insoluble calcium soaps to soluble sodium soaps by the action of sodium carbonate or bicarbonate,

bile serves to produce a more complete liberation of the fatty acid (9).

Despite these conclusions regarding the action of bile, the literature contains no quantitative data on the effect of bile in making soluble a calcium soap. We, therefore, undertook to study the effect of bile on the solubility of calcium stearate. It seemed to us that if the solubility of a soap such as calcium stearate were not appreciably greater in bile than in water, the data in the literature concerning the effect of bile upon the absorption of calcium soaps may need reinterpretation. Our experiments consisted of preparing calcium stearate and determining its composition, of adding excess of this soap to various dilutions of bile of which the free fatty acid and calcium contents were known, and determining the calcium and fatty acid in solution after periods of shaking. We were able thus to determine how much of the soap had been dissolved due to the bile.

Calcium stearate was prepared from stearic acid obtained from the Eastman Kodak Company. To a solution of sodium stearate in 90 per cent alcohol was added a calculated excess of calcium chloride in 90 per cent alcohol. The calcium soap which separated was filtered with suction, and was washed thoroughly with alcohol, and then with water (10). For analysis, weighed samples of the soap were ignited in a closed furnace. The white ash was dissolved in 0.5 cc. of 10 per cent HCl, and was then treated as in the Clark-Collip modification of the Kramer-Tisdall procedure for calcium in blood serum (11). Calibrated micro burettes were used. Calcium constituted 6.47 per cent of the soap.

Before determination of the stearic acid content of the soap, stearic acid in alcohol was neutralized with NaOH so as to form the sodium soap. The solutions were acidified, and the stearic acid was extracted as described below, and titrated. This procedure was carried through repeatedly on 10 to 15 mg. portions of stearic acid until the figures for stearic acid recovered constantly ranged between 97 and 101 per cent of the amount used.

A weighed sample of the calcium soap was placed in a 200 × 25 mm. Pyrex test-tube, and to it were added 2 drops of concentrated HCl and 30 cc. of ethyl ether. The tube was shaken until the fatty acid was dissolved. 30 cc. of distilled water were added, and the tube was shaken again. After separation into two layers, all

but 1 cc. of the ether layer was transferred by use of air pressure into a 100 cc. flask. When the layers were permitted to separate sharply, no determinable amount of HCl was carried over with the ether. The extraction was carried through twice more, and the combined ether extract was evaporated to dryness at room temperature with the aid of a gentle current of filtered air. To the residue were added 7 cc. of 90 per cent alcohol, and the flask was warmed to dissolve the fatty acid. The solution, while hot, was then titrated with alcoholic NaOH which had been standardized against small amounts of stearic acid. Phenolphthalein was used. A similar extraction of HCl without added soap was carried out, and the value obtained was subtracted from the titration value for the soap. Stearic acid was thus found to constitute 94.2 per cent of the soap. Calcium stearate should contain 6.6 per cent of calcium, and 93.4 per cent of stearic acid.

The bile used in these studies was mixed bladder bile, each sample being collected from about six steers while the bodies were still warm. For calcium of bile, the Clark-Collip modification of the Kramer-Tisdall method for blood serum was used. The bile was filtered (in all cases through Whatman paper, No. 44) before addition of ammonium oxalate. For pipetting bile, 5 minutes were allowed for drainage. From each titration value was subtracted a figure which was obtained by similar treatment of bile to which no oxalate was added. The values were practically identical with values obtained by igniting 10 cc. portions of the same filtered bile with a few drops of nitric acid in platinum crucibles, and analyzing for the calcium content of the ash, as described for the soap.

In the analysis of diluted bile for calcium, 10 cc. portions of solution were used, and 2 cc. of saturated ammonium oxalate were added directly to the samples. After centrifuging for 15 minutes, the supernatant fluid was decanted, and the tube was turned upright without the usual interval for drainage being allowed. Otherwise, some calcium oxalate would have been lost. The precipitate was washed and centrifuged with 4 to 5 cc. portions of 2 per cent  $\text{NH}_4\text{OH}$  twice, each wash liquid being decanted, and precautions being taken against loss of precipitate.

For the determination of the free fatty acid in bile, ethyl ether could not be used, as it extracted acidic substances other than fatty acids. These substances were extracted less completely by

petroleum ether. To 2 cc. portions of the bile were added 2 or 3 drops of concentrated HCl, and 10 cc. of saturated  $\text{MgSO}_4$ . 20 cc. of petroleum ether (freshly distilled below  $65^\circ$ ) were added, and the tube was inverted without loss several times, to mix the contents. Corks which had been extracted with petroleum ether were used, and the tube was inverted slowly to avoid the formation of an emulsion. After separation into layers, the petroleum ether was transferred by use of air pressure into another extraction tube, and was evaporated to dryness with the aid of a current of air, at room temperature or below. This extraction of the bile was repeated twice more, and the extracts were combined. To

TABLE I  
*Recovery of Fatty Acid Added to Bile*

Amount analyzed	Fatty acid				
	In bile	Added	Present	Recovered	
cc	mg	mg	mg	mg	per cent
2	0 417	2 04	2 457	2 598	105 7
2	1 50	2 04	3 54	3 9	110 0
5	3 96	9 5	13 46	14 9	110 4
2	1 58	3 8	5 4	6 27	116 1
2	1 58	3 8	5 4	6 1	112 9
2	0 417	2 04	2 457	2 78	113 1
2	1 58	3 8	5 4	6 0	111 1
Average					111

the residue after evaporation of the petroleum ether were added 10 cc. of water, and this residue was extracted in turn with three 20 cc. portions of petroleum ether. These portions were transferred to a 100 cc. flask, evaporated to dryness, and the fatty acid was titrated as described for the soap. When greatly diluted bile was used, 20 to 40 cc. portions were taken for analysis, and  $\text{MgSO}_4$  was not used. The accuracy of the procedure was established by analyzing for known amounts of stearic acid, added as the sodium soap to bile. Recovery of the fatty acid was consistently high by about 11 per cent. It should be stated that considerable practice with the procedure was required before sources of greater error were eliminated. The results are given in Table I.

For the determination of the solubility of calcium stearate in bile solutions, portions of the filtered bile were diluted with distilled water to make up solutions 2, 4, 8, and 16 per cent by volume of bile. To 500 cc. of each of these solutions were added 500 ( $\pm 10$ ) mg. of calcium stearate. Similar dilutions of the bile were prepared to serve as controls. The flasks were shaken at room temperature, the agitation being interrupted at designated intervals to permit the removal of samples. Excess calcium soap was filtered off, and 40 cc. portions of filtrate were analyzed for fatty acid in the 2, 4, and 8 per cent dilutions, and 20 cc. in the 16 per cent dilution.

In Table II, values of the blanks were determined by extraction of the diluted bile, carried out nearly simultaneously with the extraction of diluted bile to which calcium stearate had been added. Our experience has convinced us that blanks which are calculated from analyses of undiluted bile are not trustworthy. The variation of the fatty acid recovered with dilution of bile has been determined regularly; likewise, the different fatty acid values after prolonged shaking of the solutions. Perhaps soaps were precipitated and were filtered off without corresponding amounts of calcium being removed.

It may be seen by inspection of Table II that the values for fatty acid in the 16 per cent dilutions of bile are unsatisfactory. The variations are due probably to experimental error. The averages indicate that the amounts of fatty acid dissolved by the bile solutions were almost negligible. We have, in addition to the analyses reported in Table II, about as many more analyses, all of which lead to the same conclusion.

An attempt was made to determine the approximate solubility of calcium stearate in water. To 900 cc. portions of water were added 500 ( $\pm 10$ ) mg. of calcium stearate. The flasks were shaken occasionally over 42 hours, then were shaken continuously for 1 hour, following which they were allowed to stand for 52 and 143 hours with occasional shaking. The contents were filtered, and various amounts of the filtrate, as listed in Table III, were evaporated on the steam bath and were analyzed for calcium and fatty acid.

A flask to which no calcium stearate was added served as a control. All the figures were smaller than the experimental error,

TABLE II

*Calcium and Fatty Acid in Fresh Bile and in Old Bile after Shaking with Calcium Stearate*

Dilution of bile	Hrs shaken	Calcium			Fatty acid		
		Calculated blanks	After shaking		Calculated blanks	After shaking	
			Without Ca stearate	With Ca stearate		Without Ca stearate	With Ca stearate

## In fresh bile

per cent		mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
2	1		0 19	0 20		1 25	1 1
	5	0 264*	0 21	0 21	0 28*	1 80	1 2
	21		0 20	0 30		0 43	1 7
4	1		0 48	0 46		2 00	1 9
	5	0 530*	0 44	0 46	0 56*	1 73	2 0
	21		0 40	0 50		0 50	0 8
8	1		1 45	1 45		7 20	6 9
	5	1 440*	1 39	1 44	4 32*	6 20	6 2
	21		1 43	1 58		1 50	2 0
16	1		3 05	3 25		12 70	13 7
	5	2 900*	3 03	3 23	8 64*	9 94	16 0(?)
	21		2 88	3 48		8 70	4 1

## In old bile

8	1		0 41	0 50		28 27	29 3
	3		0 44	0 46		26 67	34 1
	6	0 696†	0 42	0 38	5 25†	41 10	40 5
	21		0 70	0 62		20 70	21 0
16	1	•	1 05	1 07		60 1	62 4
	3		1 02	1 04		60 4	57 2
	6	1.39†	0 98	1 04	10 51†	61 4	66 3
	21		1 25	1 15		43 4	40 4

\* Calculated blanks in the 2 and 4 per cent dilutions of bile were based on the analyses of 13.26 mg. of calcium and 13.90 mg. of fatty acid per 100 cc. of bile. Those in the 8 and 16 per cent dilutions were based on the analyses of 18.10 mg. of calcium and 54.00 mg. of fatty acid per 100 cc. of bile. Single determinations were run on fatty acid controls; duplicates were run on all others.

† Based on analyses of 8.7 mg. of calcium and 65.7 mg. of fatty acid per 100 cc. of bile. The bile had been kept 2 weeks at 3°.

but they can be used as evidence that the figures obtained with bile were significant. If the average value for calcium is used to calculate the solubility product of calcium stearate in water, the product  $(\text{Ca}) \times (\text{stearate ion})^2 = 3.61 \times 10^{-15}$ .

The writers recognize that changes in the reaction of bile would probably bring about changes in the solubility of calcium soaps, and that other factors, such as the bicarbonate ion concentrations, might well be studied. Because of lack of time, these questions have not been investigated.

TABLE III  
*Solubility of Calcium Stearate in Water*

Room temperature averaging 23° prevailed.

Calcium					Fatty acid				
Sample No.	Hrs.	Amount analysed	Found	Average	Sample No.	Hrs.	Amount analysed	Found	Average
		cc.	mg. per cent	mg. per cent			cc.	mg. per cent	mg. per cent
Control	95	110	0.020	0.0176	Control	95	250	0.139	0.233
"	185	100	0.018		"	95	250	0.064	
"	185	100	0.015		"	185	200	0.466	
1	95	110	0.092	0.079	4	95	250	0.195	0.535
2	185	100	0.075		5	185	200	0.700	
3	185	100	0.070		6	185	200	0.693	
					7	185	200	0.552	

#### SUMMARY

Calcium stearate of known composition was shaken with various dilutions of fresh beef bile. Portions of the mixture were withdrawn at intervals and were analyzed for the calcium and fatty acid in solution.

Under the conditions used in these experiments, bile does not dissolve appreciable amounts of calcium stearate.

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## KETOSIS DURING FASTING IN ESKIMOS

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(Received for publication, October 4, 1932)

In 1928 (1) and again in 1931 (2) evidence was presented indicating that fasting Eskimos show a lesser degree of ketosis than normal persons living in the temperate zone. The period of fasting in 1928 was of 72 hours duration; in 1931 in two subjects it was 96 hours, in two others, 115 hours. The results showed that some measure of ketosis developed in all subjects, but was only appreciable in two lactating women, one of whom was also pregnant. The fasting periods were relatively short and it was realized that if the opportunity should present itself<sup>1</sup> it would be advisable to secure data covering longer fasts. There existed the possibility that Eskimos would then show the same degree of ketosis as persons do here. The present report contains the data secured from the analysis of urine samples<sup>2</sup> collected from three persons during a 7 day fast.

The experiment was conducted at Pangnirtung, Baffin Island (latitude 66°). Three full blooded Eskimo women (Nookingah, Eto, and Martha) were chosen as subjects. Their ages were respectively 39, 18, and 14 years. Their weights were approximately 130, 120, and 110 pounds and the state of nutrition of all was similar. Prior to the experiment their diet was partly mixed because they were receiving supplies to supplement their own food from the Royal Canadian Mounted Police stores. This fact is reflected in their relatively low nitrogen output at the beginning of the fast. During the fasting period they were quartered in a recently constructed Anglican mission hospital and carefully

<sup>1</sup> Through the courtesy of the Canadian Government the author was permitted to be a member of the Canadian Arctic Expedition of 1931.

<sup>2</sup> I am indebted to Dr. P. A. Shaffer for having the urine analyses carried out in his laboratory by Mr. E. Adler.

watched. They were allowed water and tobacco. Their 24 hour urine samples were collected and measured. A 250 cc. mixed sample from each day's urine was preserved under toluene and brought to St. Louis where the samples were analyzed for acetone, diacetic acid, and hydroxybutric acid by the Shaffer-Marriott method and for total nitrogen by the Kjeldahl method. Creati-

TABLE I  
*Qualitative and Quantitative Urine Analyses*

The reaction of the urine was acid to litmus in each instance.

Subject	Day of fast	24 hr urine volume	Total N	Total acetone bodies in urine as acetone	Creatinine	Creatine	Total reducing sugars in urine	
							Fermentable	Non-fermentable
		cc	gm	gm	gm	gm	mg	mg
Nookingah	2	1949	9 34	0 0752	1 031	0 129	0 0	160 0
	3	2575	8 53	0 1391	0 805	0 325	0 0	171 0
	4	1690	11 30	0 2213	1 056	0 587	1 5	223 0
	5	525	5 03	0 3803	0 591	0 197	4 3	130 2
	6	1230	11 65	1 984	1 230	0 530	3 7	289 8
	7	1250	11 76	3 455	1 159	0 547	8 0	298 0
Eto	2	2210	9 35	0 0562	0 752	0 016	351 5	132 5
	3	2995	7 98	0 1670	0 789	0 211	37 8	132 5
	4	2055	11 42	0 1367	0 918	0 289	2828 8	266 2
	5	1050	5 41	0 0473	0 694	0 084	48 2	142 8
	6	1040	5 96	0 0907	0 694	0 036	22 9	124 9
	7	1250	5 34	0 0906	0 511	0 127	5 8	118.6
Martha	2	1210	13 92	2 824	0 756	0 652	34 0	316 0
	3	1330	13 25	0 1196	0 740	0 845	17 0	227 0
	4	1435	18 30	2 337	1 025	1 150	24 0	386 0
	5	565	8 60	0 0684	0 621	0 195	32 5	173 5
	6	700	8 42	0 1033	0 513	0 127	49 5	137 3
	7	1235	12 10	0 1348	0 680	0 092	112 1	104 7

nine and creatine were determined by Folin's method. Fermentable and non-fermentable sugars were determined according to the method of West, Scharles, and Peterson (3) modified for urine.

*Urine Analyses*—The results of the determinations are shown in Table I.

## DISCUSSION

The results of the urine analyses are consistent with those reported on two previous occasions. Accidentally the first day's urine samples were discarded, but adequate evidence has been secured on two previous occasions to indicate that normally the Eskimo shows no ketosis. The first subject, Nookingah, developed a degree of ketosis during the first 4 fasting days which is definitely below an average standard for persons of our own race and latitude. There is a pronounced increase on the 6th and 7th days to a level approximating more nearly that reported by previous investigators (4, 5). The total volume and the creatinine determinations for the 5th day point to a probable loss of urine. Even if it be assumed that the actual volume secreted was double the amount collected, the total acetone bodies for that day would be only 0.7606 gm. The ketonuria in this subject may roughly be said to have doubled in intensity daily throughout the fasting period.

Eto's results show more strikingly the ability of the Eskimo to fast without the development of any appreciable ketosis. If the creatinine determinations be taken as an index, the collected urine volumes of this subject are satisfactory. The definite increase in fermentable urine sugar on the 4th day suggests a breaking of the fast. The transgression, if it occurred, must of necessity have been slight because the total acetone bodies for the day are increased over those of the previous day. The increase in total nitrogen for the same day is no greater in this subject than it is in the other subjects at this period of the fast. The low values for fermentable urine sugars during the remainder of the experimental period indicate that the subject was then fasting.

In the case of Martha, the very definite ketosis shown by her on the 2nd and 4th days may seem an unusual finding, but it must be realized that the total number of subjects studied (ten) in the 3 years has been small. The lessening of the degree of ketosis on the 5th, 6th, and 7th days with an accompanying diminution in the nitrogen excretion seems to warrant the interpretation that this subject's ability to oxidize fats to completion improved during the fasting period. It may be assumed that the total metabolism changed but little.

Respiratory data were not secured on this occasion. If the results of 1928 and 1931 be accepted as more or less typical, it is highly improbable that the present fasting subjects were oxidizing sufficient carbohydrate to prevent the appearance of appreciable ketosis after the 3rd or 4th day of fasting. In 1931 the ketogenic to glucose ratio was found greater than 2:1 on the 3rd and 4th fasting days, with very slight ketosis. The ratio was greater on the 4th than on the 3rd day in all subjects and there is no reason to suppose that it would not increase still more on longer fasting. In two subjects of this series prolonged fasting did not result in any appreciable increase in ketosis. On previous occasions no obvious correlation between the magnitude of the ketogenic to glucose ratio and the measure of ketosis was found. In this series the first subject developed a much greater degree of ketosis than the other two. It seems, therefore, that the power to oxidize fats to completion is limited even in Eskimos. There is some variation among normal subjects. It seems consistent with our experimental finding of this and previous years to consider that in Eskimos, and probably in human beings generally, the organism possesses the ability to oxidize a certain quantity of fats to completion beyond which the oxidation becomes incomplete. Glucose, when available for oxidation, limits the demand for the oxidation of fat, thus tending to keep the subject above the threshold of ketosis.

#### SUMMARY

The analyses of urine specimens collected from three Eskimo subjects during a 7 day fast demonstrate that these people develop a lesser degree of ketosis on fasting than do average white persons living in the temperate zones.

The experimental findings on three successive fasting groups of Eskimos seem to indicate that Eskimos can burn considerable fat to completion without the aid of carbohydrate.

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# A BIOCHEMICAL STUDY OF THE FERMENTATION OF RARE SUGARS BY MEMBERS OF THE COLON- AEROGENES GROUPS OF BACTERIA\*

## I. TREHALOSE

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(Received for publication, October 12, 1932)

### INTRODUCTION

Harden (1) was among the first to study completely the end products formed when glucose was acted upon by *Escherichia coli* and *Aerobacter aerogenes*. Since Harden's publication, several workers have studied the fermentation of some of the common sugars by these two groups of bacteria. These include Harden and Walpole (2), Ayers and Rupp (3), and Virtanen and Simola (4). Similar investigations with the pentose-fermenting bacteria have been conducted by Fred, Peterson, and coworkers (5) and Gayon and Dubourg (6).

No quantitative work on the end products of fermentation in media containing the rare sugars has been reported. The object, therefore, of the research reported in this paper was to study quantitatively the fermentation products formed by the members of the colon and *aerogenes* groups of bacteria in a medium containing trehalose.

### *Methods and Procedure*

The cultures used in this study were members of the *Escherichia* and *Aerobacter* groups of bacteria. There were twenty-six cultures belonging to the former group and sixteen belonging to the latter group.

The medium for the determination of the hydrogen ion concen-

\* Presented before the meeting of the American Chemical Society at Denver, August, 1932.

tration and the qualitative gas production was prepared by dissolving 1 gm. of trehalose and 8 gm. of Bacto-Nutrient broth in 1 liter of water. This was adjusted to pH 7 and placed in Durham fermentation tubes, 10 cc. in each tube, and sterilized at a pressure of 10 pounds on each of 2 successive days.

For the quantitative determination of the products of fermentation, a medium was used which was made by dissolving 5 gm. of trehalose and 8 gm. of Bacto-Nutrient Peptone in 1 liter of water. This medium in 100 cc. amounts was placed in 250 cc. flasks and sterilized. Also, 50 cc. amounts were placed in Smith fermentation tubes and properly sterilized. Each of these tubes was previously fitted at the top of the gas arm with a small bore glass tube, which was sealed with a short piece of rubber tubing containing a plug of solid glass rod.

#### EXPERIMENTAL

Inoculations were made into the Durham fermentation tubes with the different organisms. These were incubated at 37° and gas readings and pH values were determined at 8, 24, 48, 96, and 144 hours. The pH determinations were made by means of a LaMotte hydrogen ion concentration apparatus especially designed for small amounts of liquid. The values obtained were very similar to those given later for representative organisms, so are not listed here. The members of the colon group averaged about 20 per cent gas at the end of 48 hours, whereas the *aerogenes* group produced about 30 per cent.

In addition to the pH values mentioned above, determinations were made at frequent intervals with the hydrogen electrode potentiometer in media containing 0.1 and 0.5 per cent trehalose. Four organisms were used: *Aerobacter aerogenes*, *Escherichia coli*, *Escherichia formica*, and *Escherichia communior*. The values for the last two organisms were practically identical with *Escherichia coli*. The average values for three strains each of *Aerobacter aerogenes* and *Escherichia coli* are given in Figs. 1 and 2.

The pH values and gas readings gave practically the same results for both the colon and *aerogenes* groups of bacteria. The pH values in the medium containing 0.1 per cent sugar reached a minimum in about 7 hours, and thereafter the value gradually increased until it reached a maximum of about 8.7. The mini-

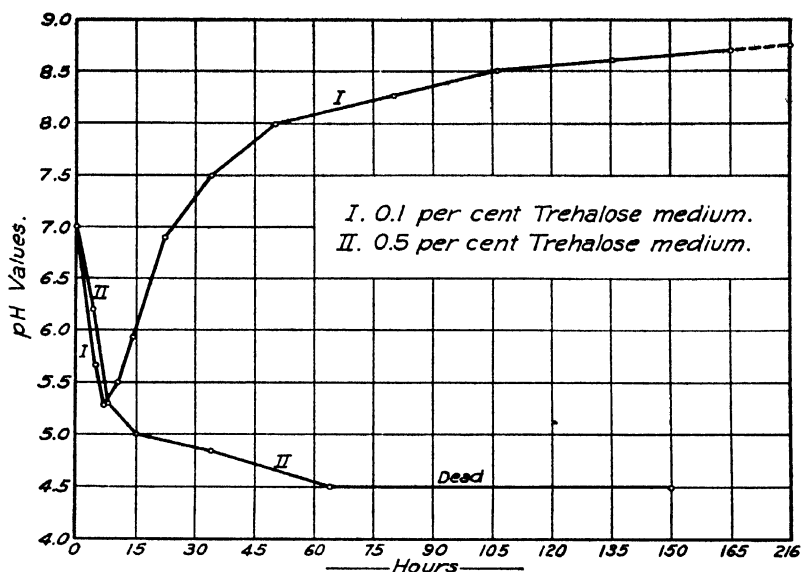


FIG. 1. Curves showing the change in pH values when *Escherichia coli* is grown in trehalose media. The broken lines represent a different interval of time.

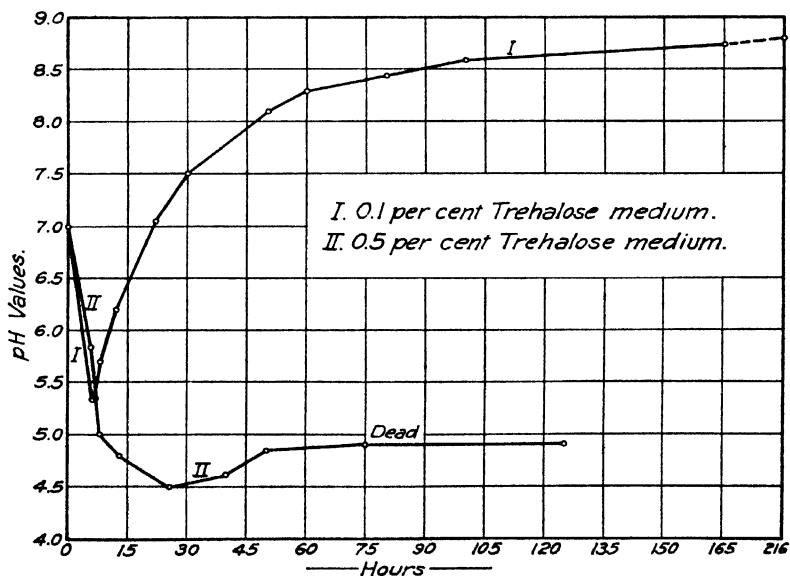


FIG. 2. Curves showing the change in pH values when *Aerobacter aerogenes* is grown in trehalose media. The broken lines represent a different interval of time.

TABLE I  
Fermentation Products in Trehalose Medium

Sample No	<i>Escherichia coli</i>		<i>Escherichia formica</i>		<i>Escherichia communior</i>		Average
	37	176	8	184	3	50	
	cc	cc	cc	cc	cc	cc	cc
CO <sub>2</sub> *	9	9	12	4	15 5	10 0	9 9
H <sub>2</sub> *	32	30	36	16	49	31	32 3
Volatile acid†	14 6	14 0	12	13 2	13 8	16 0	13 9
	mg	mg	mg	mg	mg	mg	mg
Formic “ ‡	Trace	Trace	Trace	1	None	Trace	Trace
Acetic “	88	84	72	79	83	96	83 7
Succinic “	53	58	57	50	61	61	56 7
Lactic “	27	33	24	30	21	20	25 8
Ratio, volatile/ non-volatile	1 10	0 92	0 89	1 00	1 01	1 18	1 02
Acetic/succinic	1 66	1 45	1 26	1 58	1 36	1 57	1 48
“ /lactic	3 26	2 55	3 00	2 63	3 95	4 80	3 37
Alcohol	1000	610	606	210	812	807	674
	<i>Aero- bacter aerogenes</i>	<i>Aero- bacter oxyzotum</i>	<i>Aero- bacter chinense</i>	<i>Aero- bacter leians</i>	<i>Aerobacter cloacae</i>		
Sample No	123	91	227	161	5	100	
	cc	cc	cc	cc	cc	cc	cc
CO <sub>2</sub> *	67	31	13	26	64	31 5	38 8
H <sub>2</sub> *	94	49	27	38	96 0	48 5	58 7
Volatile acid†	17 8	19 8	18 2	18 6	17 4	17 7	18 2
	mg	mg	mg	mg	mg	mg	mg
Formic “ ‡	1	1	1	None	None	1	0 7
Acetic “	105	118	109	112	104	105	108 8
Succinic “	25	28	26	25	32	31	27 8
Lactic “	51	41	45	47	36	36	42 7
Ratio, volatile/ non-volatile	1 39	1 72	1 55	1 56	1 53	1 58	1 55
Acetic/succinic	4 20	4 21	4 19	4 48	3 25	3 39	3 95
“ /lactic	2 06	2 88	2 42	2 39	2 89	2 92	2 59
Alcohol	1040	1010	1100	1200	1150	1380	1147

\* Volume formed in a Smith fermentation tube

† Amount of 0.1 N alkali used to neutralize the acid from 1 gm. of sugar.

‡ All of the values in mg are on the basis of 1 gm of trehalose.

num was much lower for the medium containing 0.5 per cent sugar; the acidity developed, however, killed the organisms in about 60 hours.

The Smith fermentation tubes and flasks containing the 0.5 per cent sugar medium were inoculated and incubated for 72 hours at 37°. The gases and other fermentation products were analyzed and the results are given in Table I.

The amounts of the products formed with the two groups of bacteria show considerable difference. The *aerogenes* group produced a larger amount of each constituent except the succinic acid. The colon group formed twice as much of this particular acid as did the *aerogenes* group. Another significant difference is found in the acid ratios. The ratio for acetic to succinic acids was greater for the *aerogenes* group and less for the acetic to lactic acids. The reverse was true for the colon group. All of the members of the *aerogenes* group gave tests for acetylmethylcarbinol, whereas those of the colon group did not.

#### SUMMARY

1. The fermentation products by members of the colon and *aerogenes* groups of bacteria in trehalose medium have been determined.

2. The progressive changes in pH values during fermentation have been studied.

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# A SOXHLET TYPE OF EXTRACTION APPARATUS FOR OPERATION AT LOW TEMPERATURES UNDER REDUCED PRESSURE

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The Soxhlet type of fat extraction apparatus is not well adapted to the extraction of thermolabile biological products, as the extracted matter is subjected to the boiling temperature of the solvent used. Ethyl ether, which has a conveniently low boiling point, is unsuited for many extraction purposes (1). In the course of a serological examination of serum lipids, the Soxhlet type of apparatus was found to give the most satisfactory extraction, but to permit the use of a wider range of solvents the apparatus was modified to operate under reduced pressure. This allowed the boiling point of the solvent, and hence the temperature during the extraction, to be adjusted as desired. The apparatus described herewith has been in use for 2 years, and has proved very satisfactory.

The first apparatus which we constructed for extraction under reduced pressure had tapered ground joints which were made airtight by a mercury seal. After holding the apparatus under a vacuum, these ungreased joints were difficult to separate. It is not feasible to grease the joints, since the organic solvents used would dissolve some of the grease and contaminate the extract. Nor can rubber stoppers be used at the joints without contamination of the extracts. This led us to use flat glass joints, which have met our requirements. The complete apparatus is shown in Fig. 1, and is constructed entirely of Pyrex glass. The bulb condenser is 30 inches long, as a large condensing surface is needed when working at lower temperatures under reduced pressure.

\* Aided by the National Research Council of Canada.

## 290 Soxhlet Type of Extraction Apparatus

The condenser is supported by rubber-padded clamps attached to a  $\frac{1}{2}$  inch iron rod, which runs from the top of the bench to the ceiling. There is only one joint in the apparatus, where the flask fits the condenser. This joint, as shown in Fig. 1, is made perfectly flat, finely ground, and then highly polished. The amount of air which leaks in through a well made joint of this type is very small. In operating this apparatus at absolute pressures of 100 to 200

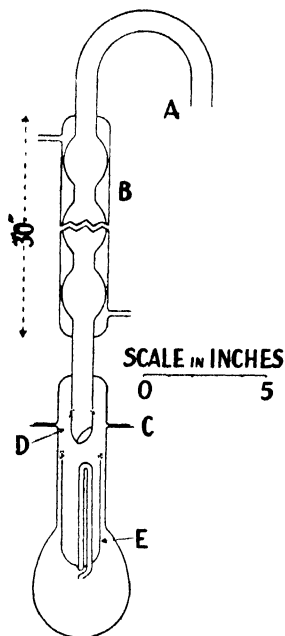


FIG. 1. Complete extraction apparatus. *A* is a pipe leading to the vacuum line; *B*, a reflux condenser; *C*, a flat joint between flask and condenser; *D*, a wire which passes through small holes on opposite sides of the vapor pipe and supports the siphon extraction cup, *E*.

mm. of Hg, the leakage amounts to 20 to 30 cc. of outside air per minute. For most work this is of no significance, although in extracting substances which are very subject to oxidation it is desirable to have the apparatus completely air-tight. This can be largely achieved by slightly changing the joint as follows: The joints are finely ground, but not polished, the flat surface of the flask being slightly larger than the corresponding flat surface

attached to the condenser (Fig. 1). The apparatus is connected with the flat joints clean and dry, and melted paraffin wax is then painted around the outside edge of the joint where it at once solidifies. When using this method, all our extractions were carried out at 50° or lower, so that no softening of the wax occurred. Although paraffin wax is soluble in many of the organic solvents employed, contamination of the extract with traces of paraffin was very rare. This method of sealing the joints is not ideal, and the dry polished joints are always to be preferred where the slight leakage of air into the apparatus will not impair the material which is being extracted. One great advantage of the flat polished joint over the tapered joint is that a number of flasks may be used

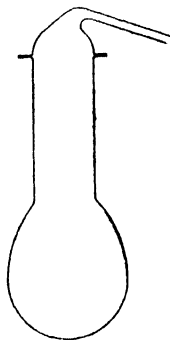


FIG. 2. Apparatus used for concentrating the extracts by boiling off most of the solvent under reduced pressure. The same flask which is used during the extraction (see Fig. 1) may be used here.

interchangeably, and additional flasks can be made by the glass-blower without having the condenser available. The same type of joint is equally satisfactory for the preliminary concentration of the extracts, as indicated in Fig. 2.

To avoid the slight warping which may take place in glass soon after being worked in the flame, it is advisable that after making and rough grinding the flat joint, the apparatus should be laid aside for 2 or 3 weeks before the final grinding and polishing of the joint. The polished joints on our apparatus are  $\frac{1}{2}$  inch wide. Fig. 1 shows the 1 inch wide unpolished joint which was used when the edge of the joint was sealed by paraffin wax.

The neck of the extraction flask is 2 inches in diameter, which

## 292 Soxhlet Type of Extraction Apparatus

allows the use of extraction thimbles  $1\frac{1}{4}$  inches in diameter. It is not advisable to construct the apparatus for flasks greater than 2 inches in diameter at the neck, as it is difficult or impossible to grind and polish such large joints with the desired accuracy.

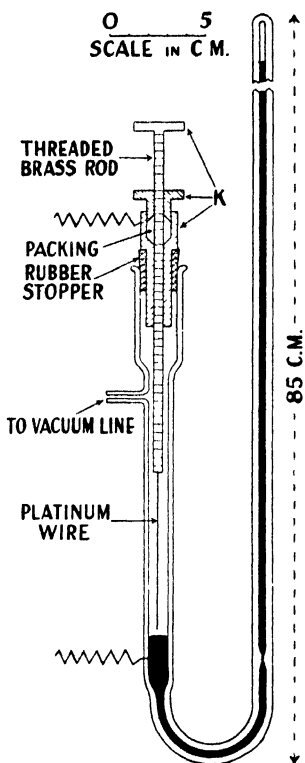


FIG. 3. Manometer to maintain desired degree of vacuum. The top nut of the packing gland, which is threaded onto the brass rod, is loosened when the brass rod is being screwed up or down. The metal faces marked *K* are knurled to provide easier grip for the fingers. The manometer is mounted by means of putty into a suitable channel gouged out of a thick wooden board.

We have found this apparatus easier to operate than the ordinary Soxhlet extractor. On holding the flask in position below the condenser and opening the tap to the vacuum line, the flask is held firmly in place by air pressure, and no clamp should be used.

The electric heater should be covered by a piece of rather soft  $\frac{1}{4}$  inch asbestos board, the hole in the asbestos being of such size that the flask is heated only below the liquid level. The heater must always be removed before the vacuum is broken.

The vacuum may be held at any desired value by means of an automatic manometer such as is shown in Fig. 3. The long arm of the manometer tube is of 3 mm. bore, while the shorter limb is of 9 mm. bore. In the wall of this shorter limb is sealed a platinum wire to make a permanent contact with the mercury, while an adjustable platinum contact wire is attached to a threaded brass rod working in an air-tight packing gland. This contact can be adjusted to give any desired degree of vacuum, from air pressure to 1 cm. absolute pressure. The small current passing through the Pt-Hg contact in the manometer operates a relay mercury switch which starts and stops the motor for the vacuum pump. The pump is connected to a heavy 10 gallon glass bottle which acts as a vacuum reserve and stabilizer, and to this bottle also runs the vacuum line from the extraction apparatus. The pump should not be connected directly to the vacuum line running to the extraction apparatus, as this would cause larger fluctuations of pressure.

Most of our work with this apparatus has been carried out at temperatures of 35–45°, and extraction at these lower temperatures is satisfactory, provided that the sample is thoroughly dried upon the inert base. We have found finely ground  $\text{Na}_2\text{SO}_4$  to be a suitable base, while  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  has always been unsatisfactory, as Table I indicates. These figures are for an extraction of 40 minutes at 40° with absolute ethyl alcohol.

TABLE I  
*Efficiency of Extractions*

Inert base	Solvent	Fatty acid extracted per 100 cc serum	Efficiency of extraction
		<i>mg</i>	<i>per cent</i>
$\text{Na}_2\text{SO}_4$	$\text{C}_2\text{H}_5\text{OH}$	103 4	99 3
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	"	73 5	70 6

The sheep serum used contained 104.2 mg. of total fatty acid per 100 cc. by Bloor's method (2).

The vapor pressures of all the common solvents over a wide range of temperature are listed in the chemical handbooks (3).

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## THE USE OF THE CHICK IN VITAMIN B<sub>1</sub> AND B<sub>2</sub> STUDIES\*

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The chicken was used as an experimental animal very early in the history of vitamin research. Eijkman (1), using this animal, demonstrated in 1897 the existence of an antineuritic substance obtainable from rice polishings, and indispensable to the growth and health of the fowl. Grijns, Vedder and Clark, and Fraser and Stanton also used the chicken for beriberi studies. This early work is reviewed by Funk (2).

Since the food requirements of pigeons were found to be much simpler than those of chicks, and because beriberi was readily produced in this animal, the pigeon replaced the chick to a large extent in vitamin B studies. The early work with pigeons is also reviewed by Funk (2).

After McCollum and Kennedy (3) demonstrated that those natural foods which cured polyneuritis in chickens and pigeons also promoted growth in rats fed a vitamin B-deficient diet, the rat was used extensively as the experimental animal. This animal has been used almost exclusively in the investigations concerning the dual nature of vitamin B.

Emmett and Luros (4) and Levene and Muhlfeld (5) used both rats and pigeons in their attempts to prove that the antineuritic factor for pigeons and the growth-promoting factor for rats are not identical. In 1926 Hauge and Carrick (6) used the chicken exclusively for the differentiation between the water-soluble growth-promoting factor and the water-soluble antineuritic substance. Hauge and Carrick used the rate of growth of the chickens for evaluating the second factor of the vitamin B complex. In

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1930 Norris and Ringrose (7) described a pellagrous-like syndrome in chicks which could be prevented by the addition of autoclaved yeast which is rich in the growth-promoting factor.

Williams and Waterman (8) used the pigeon for demonstrating that the vitamin B complex contains a third, highly thermolabile factor necessary for maintenance of weight and general condition of adult pigeons. Further evidence was presented by Eddy, Gurin, and Keresztesy (9) substantiating the existence of a bird growth and weight restoration factor designated by Williams and Waterman as B<sub>3</sub> and distinct from vitamins B<sub>1</sub> and B<sub>2</sub>. Eddy and coworkers showed that this factor is also necessary for the chick.

Carter, Kinnersley, and Peters (10) accepted the B<sub>3</sub> factor studied by Williams and Waterman and by Eddy, Gurin, and Keresztesy, and concluded that the pigeon requires for maintenance nutrition an additional factor, designated as vitamin B<sub>5</sub>, which is distinct from vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and B<sub>4</sub>.

From the work of Williams and Waterman, Eddy, Gurin, and Keresztesy, and Carter, Kinnersley, and Peters it is necessary to conclude that of the five vitamin B factors which have been studied, the pigeon needs vitamins B<sub>1</sub>, B<sub>3</sub>, and B<sub>5</sub> and the rat needs vitamins B<sub>1</sub>, B<sub>2</sub>, and B<sub>4</sub>. Only one component, the anti-neuritic vitamin B<sub>1</sub>, is required in common by the two species. The results which we have obtained *with chickens* indicate that the heat-labile vitamin B<sub>3</sub> factor is not necessary for this species, but that vitamin B<sub>2</sub> is required in relatively large amounts. While carrying out these experiments, methods for rendering a ration of natural foodstuffs deficient in vitamins B<sub>1</sub> and B<sub>2</sub> were developed. A description of the preparation and use of these rations will be given in this paper.

#### EXPERIMENTAL

For these experiments day-old white Leghorn chicks with an initial weight of 30 to 35 gm. were procured from the University Poultry Department or from commercial hatcheries. They were placed in individual cages (14 × 16 × 14 inches) in order to allow the recording of individual food consumption. The cages were individually heated and equipped with raised wire screen (three meshes to the inch) floors.

During the first 2 weeks of the experiment the ration was placed in small, round Daton feeders (No. 1015). Five of the six holes were stoppered with corks and an additional hole made in the center, which left two openings through which the chicks could consume the ration. This type of feeding served to minimize the loss of food due to scratching and spilling.

At 2 weeks of age, the small feeders were replaced by  $2 \times 3 \times 10$  inch feed pans equipped with  $\frac{1}{2}$  inch flanges to prevent the scattering of feed. Each chick was separated from the feeding pan by means of two wire grids, superimposed, and sliding one upon the other so that the openings could be made just large enough for the chick to place its head through the grid. The feed pan was then placed flush with the opening of the cage and the grids immediately behind to allow the chick as much space as possible. The water cup was placed a short distance from the feed pan to avoid the splashing of water into the food.

The basal ration formulated for use in these experiments consisted of the following ingredients.

Yellow corn.....	58	Salt (common).....	1
Middlings.....	25	Cod liver oil.....	2
Casein.....	12	CaCO <sub>3</sub> .....	2

The yellow corn used was obtained directly from the field and dried at room temperature (20°). The middlings were standard wheat middlings and the casein was a commercial preparation. This ration will hereafter be designated as Ration 240.

The ability of this ration to promote normal growth of the chick was demonstrated in preliminary experiments. To render this ration deficient in vitamins B<sub>1</sub> and B<sub>2</sub>, it was subjected to various heat treatments. In this procedure only the yellow corn, middlings, casein, and salt were treated thermally; the CaCO<sub>3</sub> and cod liver oil were added to the heated portion as the rations were prepared for feeding each week. The feed was weighed into the pans at the beginning of each week. At the end of the week the pans were reweighed, the ration not consumed discarded, and the pans refilled. In some cases it was necessary to make further weighed additions during the week. Weekly consumption records have been kept throughout all our experiments.

Since forty-eight individual cages were available for the work,

it was possible to study at one time twelve different rations with four chicks on each ration. However, two groups were always used for positive and negative controls in each series. In this paper no attempt will be made to present a complete record of all the experimental work; only those results will be given which are needed to demonstrate that vitamins B<sub>1</sub> and B<sub>2</sub> can be destroyed independently in a natural ration. In all cases the growth curves for four individual animals on any specific ration will be given. In most cases many more animals have been reared on the same ration but the four curves are typical of all the animals.

When chicks are placed on the basal unheated ration (Ration 240) good growth is obtained and the animals appear normal in every way. The growth curves are given in Chart I. The growth is comparable to that observed for white Leghorn chicks grown on any complete ration. Chicks placed on a ration identical in all respects except that it had been autoclaved at 120° and 15 pounds pressure for 5 hours failed to grow normally and developed polyneuritis at about 8 days of age. Growth curves for these chickens are also given in Chart I. The development of polyneuritis in all chicks receiving this ration was exceedingly uniform, usually not before 7 days and not later than 10 days of age. The animals became extremely emaciated before death and exhibited a general lack of muscular coordination and sense of balance. This condition usually lasted about 1 day before the typical opisthotonos of the neck muscles was observed. Death followed shortly after these characteristic symptoms developed. Fig. 1 illustrates a bird exhibiting typical vitamin B<sub>1</sub> deficiency. Ration 240 which has been autoclaved at 120° for 5 hours and which produces this typical vitamin B<sub>1</sub> deficiency will be designated as Ration 240-A.

This condition was cured by the administration of yeast or was prevented by incorporating 4 per cent of air-dried yeast in the ration. The dried yeast was prepared from fresh bakers' yeast which was obtained from the Red Star Yeast Company, Milwaukee, and dried immediately at 65°. The results in Chart I show that 2 per cent of this yeast was insufficient to prevent polyneuritis but that 4 per cent was ample for complete protection. The chicks receiving 8 per cent showed no improvement over those getting 4 per cent yeast. These results demonstrate that vitamin B<sub>1</sub> is destroyed in a ration of natural foodstuffs by autoclaving

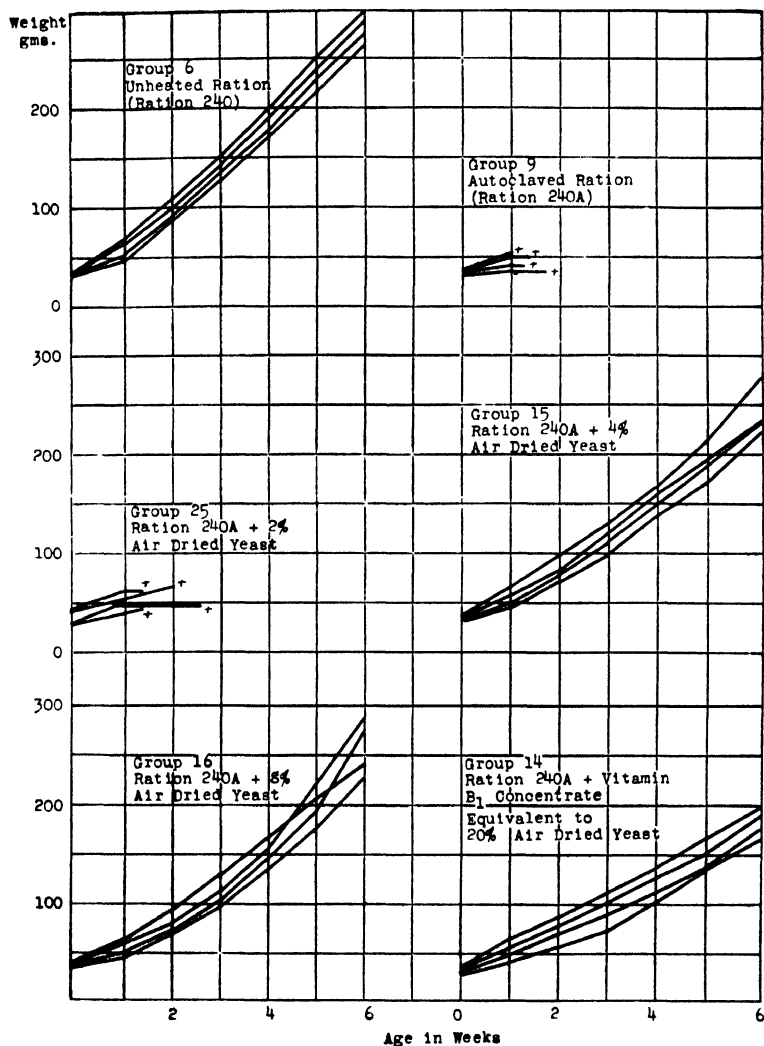


CHART I. Typical growth records of chicks reared on the unheated ration (Ration 240) and on the autoclaved ration (Ration 240-A) with and without vitamin B<sub>1</sub> supplements. The chicks receiving Ration 240-A and Ration 240-A plus 2 per cent of air-dried yeast exhibited typical polyneuritis before death.

and that the deficiency can be prevented by the addition of 4 per cent of air-dried yeast. They do not eliminate the possibility that other factors are also destroyed by this treatment and in turn supplied by the yeast. However the administration of a vitamin B<sub>1</sub> concentrate prevented polyneuritis and allowed growth.

The vitamin B<sub>1</sub> preparation was made as follows: 2000 gm. of fresh bakers' yeast were suspended in 1000 cc. of boiling water acidified with 1 cc. of glacial acetic acid, and boiled 5 minutes with constant stirring. The yeast was filtered off and reextracted under the same conditions. The filtrates were combined and adjusted



FIG. 1

FIG. 1. Typical opisthotonos of the neck muscles exhibited by chicks suffering from vitamin B<sub>1</sub> deficiency.



FIG. 2

FIG. 2. Scabby incrustations of the corner of the mouth and lower mandible exhibited by pellagic chick.

to pH 4.5 with HCl, and 80 gm. of fullers' earth were added. The suspension was stirred frequently and allowed to stand overnight. The fullers' earth was filtered off and the filtrate treated with an additional 40 gm. of fullers' earth. After standing 6 hours the suspension was filtered and the two samples of fullers' earth were combined and dried at room temperature. 1 gm. of activated fullers' earth was equivalent to 16 gm. of fresh yeast.

The results obtained when four chicks were fed the autoclaved ration (Ration 240-A) plus the B<sub>1</sub> preparation equivalent to 20 per cent of air-dry yeast are given in Chart I. The good growth indicates that vitamin B<sub>1</sub> is the only factor destroyed during autoclaving.

Since a ration compounded from grains and grain products may be low in vitamin B<sub>2</sub>, the effect of additional amounts of this factor in the form of autoclaved yeast was studied. The growth curves for four chicks raised on the autoclaved ration plus 4 per cent autoclaved yeast and 4 per cent air-dried yeast are given in Chart II. The growth of these chicks is no different from that of

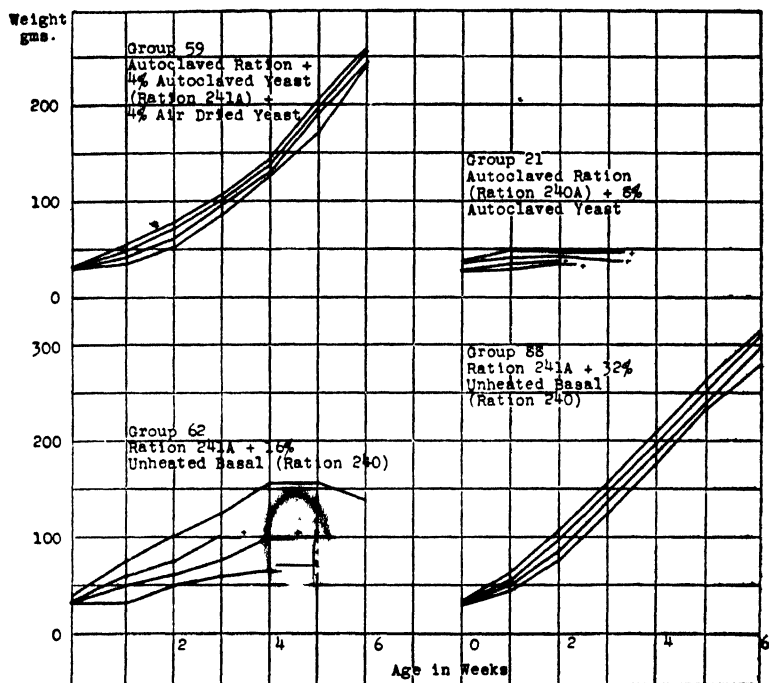


CHART II. Growth records of chicks raised on autoclaved ration plus additional vitamin B<sub>2</sub> in the form of autoclaved yeast with and without vitamin B<sub>1</sub> supplements.

those receiving 4 per cent of air-dried yeast alone. It is readily seen from the growth records of the chicks fed 8 per cent of autoclaved yeast that autoclaved yeast furnishes no vitamin B<sub>1</sub>. The autoclaved ration can therefore be used for testing the vitamin B<sub>1</sub> potency of materials containing large amounts of vitamin B<sub>2</sub>.

The results obtained when Ration 240-A was used for testing the vitamin B<sub>1</sub> potency of the original unheated ration are given in

Chart II. The chickens receiving 16 per cent of the ration were protected to some extent, while those receiving 32 per cent were protected completely and grew normally. These results show that the natural ration contains about one-eighth as much vitamin B<sub>1</sub> as the yeast used. In much of our work we have used Ration 240-A plus 4 per cent autoclaved yeast as the basal ration in order to insure a plentiful supply of vitamin B<sub>2</sub>. The ration containing autoclaved yeast has been designated as Ration 241-A. Either of these rations is well suited for testing the vitamin B<sub>1</sub> potency of any food material, for two reasons; first, because they are easily prepared and, secondly, because the vitamin B<sub>1</sub> activity is determined under conditions which are comparable to those under which the vitamin is generally consumed.

While testing the stability of the vitamin B factors at temperatures lower than 120°, it was found that chicks reared on the basal diet *heated dry at 100° for 144 hours* developed definite symptoms of pellagra. For the heat treatment, the ration was placed in suitable pans and allowed to remain in an oven kept at 95–100° for 6 days. To insure uniform treatment in the different batches, a  $\frac{1}{4}$  inch layer of feed was always placed in each pan. Ration 240 which has been heated dry at 100° for 144 hours is designated as Ration 240-H.

All chickens grown on this ration invariably developed pellagra at about 3 weeks of age. The symptoms are very similar to those described by Norris and Ringrose (7). As these workers state, the external manifestations appear chiefly at the eyes, the corners of the mouth, and upon the legs and feet. The feathering is retarded, and the feathers are very ruffled because the birds peck themselves continually. The crusty scabs at the corners of the mouth gradually enlarge and often involve the margins of the skin around the nostrils and underneath the lower mandible. The condition develops gradually and the animals often survive 2 weeks or more after the first appearance of the disease. The appetite does not decrease as is the case with chicks suffering from vitamin B<sub>1</sub> deficiency. The average weekly food consumption for a number of normal chicks, vitamin B<sub>1</sub>-deficient chicks, and vitamin B<sub>2</sub>-deficient chicks is given in Table I.

The growth records for four chicks on the unheated ration (Ration 240) and four chicks on the ration heated at 100° for 144 hours (Ration 240-H) are given in Chart III. It is readily seen

TABLE I

*Average Weekly Consumption Records (in Gm.) of Normal, Vitamin B<sub>1</sub>-, and Vitamin B<sub>2</sub>-Deficient Chicks*

Wk.	Ration 240; basal, unheated	Ration 240-A; basal, autoclaved; vitamin B <sub>1</sub> -deficient	Ration 240-H; basal, heated at 100° for 144 hrs.; vitamin B <sub>2</sub> -deficient
1	32	30	30
2	45	20	46
3	90		62
4	115		65
5	204		85
6	240		

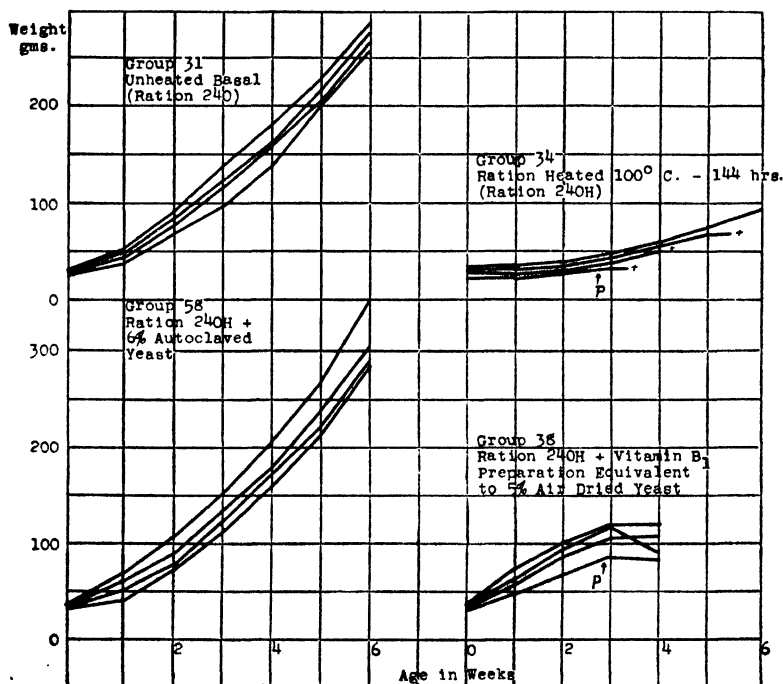


CHART III. Growth records of chicks fed Ration 240 and Ration 240 heated at 100° for 144 hours (Ration 240-H) with and without vitamins B<sub>1</sub> and B<sub>2</sub> supplements. P denotes the approximate time at which the symptoms of pellagra were first noted.

that this heat treatment develops in the ration a deficiency characterized by retarded growth and a pellagrous condition in the chick. Fig. 2 illustrates this deficiency in the chick.

When 6 per cent of autoclaved yeast is added to the ration heated at 100° for 144 hours (Ration 240-H), the development of pellagra is prevented and normal growth is obtained. The addition of a

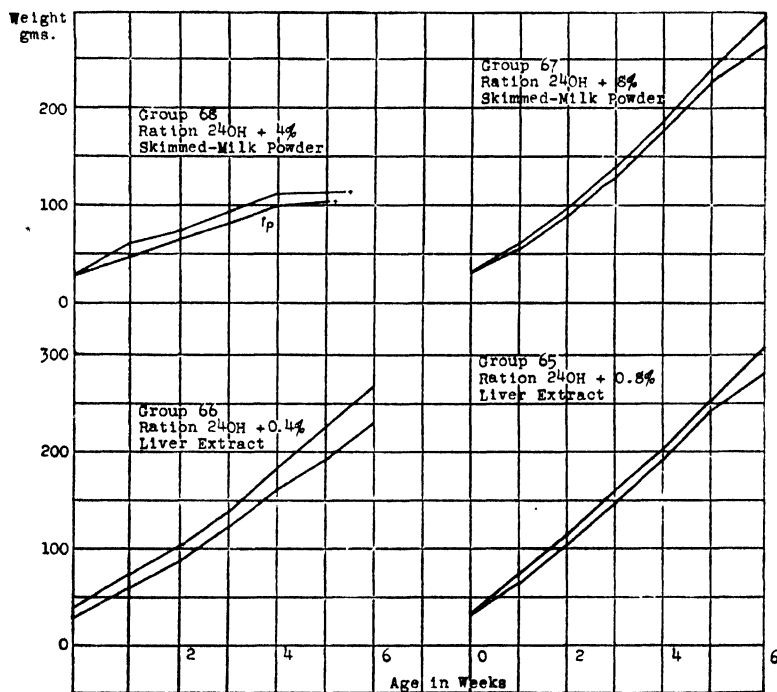


CHART IV. Growth records of chicks fed Ration 240-H supplemented with skim milk powder and liver extract (Lilly, No. 343). P denotes the approximate time at which the symptoms of pellagra were first observed.

vitamin B<sub>1</sub> preparation did not prevent pellagra and had practically no effect on growth. These results demonstrate conclusively that chicks require vitamin B<sub>2</sub> for normal development. Williams and Waterman (8) concluded, as did Seidell (11), that pigeons do not need this vitamin. However, Eddy and coworkers (9) showed that, unlike the adult pigeon, growing chicks showed

some need for vitamin B<sub>2</sub>. Our results are in complete accord with those of Norris and Ringrose who showed the very great requirement of this species for vitamin B<sub>2</sub>.

We have used Ration 240-H for testing the vitamin B<sub>2</sub> potency of a number of foodstuffs. The results obtained for skim milk powder and liver extract (Lilly, No. 343) are given in Chart IV. 8.0 per cent of skim milk powder and 0.4 per cent of liver extract supplied sufficient vitamin B<sub>2</sub> to meet the requirements of the chick.

It is possible, therefore, to destroy the vitamin B<sub>2</sub> in a ration of natural foodstuffs by heating dry at 100° for 144 hours and at the same time allow sufficient amounts of vitamin B<sub>1</sub> to remain to supply the requirement of the chick. Ration 240-H is especially valuable, because it eliminates the necessity of preparing and adding a vitamin B<sub>1</sub> concentrate to a ration deficient in both vitamins B<sub>1</sub> and B<sub>2</sub>, which is the usual procedure in vitamin B<sub>2</sub> assay.

#### DISCUSSION

In the past the majority of the workers have used either a rice diet for vitamin B<sub>1</sub> studies with pigeons or synthetic diets for vitamins B<sub>1</sub> and B<sub>2</sub> studies with rats. In the case of the rice diet no serious difficulties have been encountered because pigeons develop polyneuritis so rapidly that no other factors complicate the results; nevertheless, the rice is far from a complete diet. The use of synthetic diets has introduced many complicated problems in vitamin assay. This is especially true in the case of chickens as has been pointed out in several papers by Hogan and coworkers (12). When a synthetic ration is compounded it is difficult to determine whether the ration is deficient only in the factor to be studied or if it is lacking in other nutrients as well. Generally the ration is so constructed that one or more factors in addition to the one being investigated must be added in concentrated form. In this case it is difficult to prepare these concentrates so that they are exceedingly low in or devoid of the factor in question. The existence or non-existence of some of the recently reported factors rests upon the purity of the preparations used.

Whether workers are justified in concluding that the requirements

of an animal for a certain vitamin are the same when it is added to a synthetic diet as when added to a diet of natural food materials is a question which may be raised also. In this paper we have described methods for rendering a diet of natural foodstuffs deficient in vitamin B<sub>1</sub> or B<sub>2</sub> respectively. The original ration is compounded from materials which are ordinarily consumed by humans or farm animals and is complete in all known nutrients before it is subjected to either of the treatments for the destruction of the vitamins.

#### SUMMARY

1. A ration of natural foodstuffs (Ration 240) has been devised which can be rendered deficient in vitamin B<sub>1</sub> or B<sub>2</sub> respectively by different heat treatments.

2. When Ration 240 was autoclaved at 120° and 15 pounds pressure for 5 hours (Ration 240-A) and fed to chicks, the chicks developed severe polyneuritis at about 8 days of age. The incorporation of air-dried yeast or a vitamin B<sub>1</sub> concentrate in the ration corrected this deficiency. Ration 240-A was usually supplemented with additional vitamin B<sub>2</sub> in the form of 4 per cent of autoclaved yeast (Ration 241-A) when it was used as a basal ration for vitamin B<sub>1</sub> studies.

3. When Ration 240 was heated dry at 95–100° for 144 hours (Ration 240-H) and fed to chicks, the chicks developed pellagra at about 3 weeks of age. This ration was rendered complete by the addition of autoclaved yeast.

4. A few results obtained when Rations 241-A and 240-H were used in assaying the vitamin B<sub>1</sub> and vitamin B<sub>2</sub> content of various foods are given.

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## A STUDY OF THE HEAT STABILITY OF THE VITAMIN B FACTORS REQUIRED BY THE CHICK\*

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Vitamin B<sub>2</sub> has been considered the more heat-stable constituent of the vitamin B complex since the early work of Goldberger, Wheeler, Lillie, and Rogers (1) in 1926. In the preceding paper (2) results were presented to show that vitamin B<sub>2</sub> was destroyed in a ration of natural foodstuffs when heated dry at 100° for 144 hours. These results show that under certain conditions vitamin B<sub>2</sub> can be inactivated at a temperature lower than that required for the destruction of vitamin B<sub>1</sub>. It is evident, therefore, that further studies on the heat stability of the vitamin B fractions are necessary.

The present investigation deals mainly with results obtained when the diets described in the preceding paper and yeast were used for studying the stability of vitamins B<sub>1</sub> and B<sub>2</sub>. However, the use of these rations also afforded us some opportunity to study the possible existence of the heat-labile vitamin B<sub>3</sub> which was first described by Williams and Waterman (3) as an additional vitamin B factor required by the pigeon and later regarded to be necessary for the chick by Eddy, Gurin, and Keresztesy (4).

Since the original ration used in these studies undoubtedly contained all additional heat-stable factors required by the chick, it was impossible to study such factors as vitamin B<sub>3</sub> described by Carter, Kinnorsley, and Peters (5). However, we have studied the importance of additional factors for the chick when synthetic diets are used but this work will be reported in a later paper.

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*Heat Stability of Vitamin B<sub>1</sub>*

Most of the work concerning the stability of vitamin B<sub>1</sub> has centered around the effect of pH on the rate of destruction. Some attempt has been made to correlate the action of pH and temperature. Sherman and Grose (6) showed that the decrease in acidity from pH 4.28 to pH 5.2 increased the rate of destruction to about the same extent as did the increase in temperature from 100–110°.

The accepted method for destroying vitamin B<sub>1</sub> is autoclaving at 120° for 5 hours. Autoclaved yeast, which is free from or very low in vitamin B<sub>1</sub>, is used by practically all workers as a source of vitamin B<sub>2</sub>. There are at least two factors operative during autoclaving; namely, heat and moisture. Since the vitamin B<sub>1</sub> in a natural ration is destroyed completely when the ration is autoclaved at 120° for 5 hours, and destroyed only to a very slight extent when the ration is heated dry at 100° for 144 hours, the presence or absence of moisture must be a determining factor in inactivation.

Sherman and Spohn (7) observed some effect of moisture on the destruction of vitamin B<sub>1</sub> as early as 1923, before the importance of vitamin B<sub>2</sub> was recognized. They found no measurable diminution of vitamin B (B<sub>1</sub>) in milk powder when heated dry for 48 hours but found a destruction of about one-fourth of the total when fluid milk was heated at 100° for 6 hours. Sherman and Burton (8) suggested that the destruction of vitamin B is due to a hydrolysis or intramolecular rearrangement which is catalyzed by hydroxyl ions.

The following experiments were conducted to demonstrate the influence of moisture on the destruction of vitamin B<sub>1</sub>. Day-old white Leghorn chicks were placed in individual cages and fed Ration 241-A. The preparation of this ration is described in the preceding paper (2). Chicks grown on this ration invariably develop polyneuritis in 7 to 9 days. All the yeast preparations were made from one batch of fresh Red Star yeast. The yeast used for Groups 77 and 20 was dried at 100° for 24 hours. 4 per cent of the dry yeast was incorporated into the basal ration for Group 77 and 8 per cent was used for Group 20. Group 60 received 4 per cent yeast dried at 67° and then heated dry at 100°

for 24 hours. Group 79 was given 4 per cent yeast dried at 67° and then heated moist at 100° for 24 hours. Group 80 was fed 4 per cent of yeast dried at 67° and then heated moist at pH 3.0 for 24 hours at 100°. Group 9, receiving the basal autoclaved ration alone, is included for comparison. The growth curves for four chicks in each group are given in Chart I.

It is readily seen that moisture is an essential factor in the destruction of vitamin B<sub>1</sub>. The chicks receiving their supply of vitamin B<sub>1</sub> from yeast dried at 100° for 24 hours (Group 77) did not develop polyneuritis as early as those receiving the basal ration alone but all of them finally showed the characteristic deficiency symptoms. The addition of 8 per cent of this yeast protected the chicks from polyneuritis and produced normal growth (Group 20). This indicates that about one-half of the potency was destroyed during the heating process. Yeast dried at 100° loses its water in a few hours, but the presence of moisture even for this short time was sufficient to bring about a definite decrease in potency. These results demonstrate the importance of controlling the temperature when fresh yeast or moist foods are prepared for vitamin B<sub>1</sub> work.

When the yeast was dried first at 67° and then heated dry at 100° for 24 hours the chicks grew normally (Group 60), which shows that very little destruction takes place at 100° in the absence of moisture. The results for the chicks given yeast which had been dried at 67° and then moistened with distilled water before being heated at 100° for 24 hours are most striking. All the chicks developed severe polyneuritis when 7 to 9 days old (Group 79). The vitamin B<sub>1</sub> was destroyed completely under these conditions. The pH of the yeast mixture was approximately 6.4. The chicks which were fed yeast heated moist at pH 3.0 grew well and exhibited no signs of deficiency (Group 80). The rate of destruction is not only dependent upon the presence of moisture but also upon the hydrogen ion concentration of the solution. Guha and Drummond (9) found that the activity of a vitamin B<sub>1</sub> concentrate decreased about one-half when it was boiled at pH 5.0 for 24 hours.

Similar results were obtained when the basal ration (Ration 240) was used as the source of vitamin B<sub>1</sub> in place of the yeast. When 24 or 32 per cent of unheated ration or similar amounts of

the ration heated dry for 24 hours were added to Ration 241-A good growth was obtained, but when the ration was heated moist

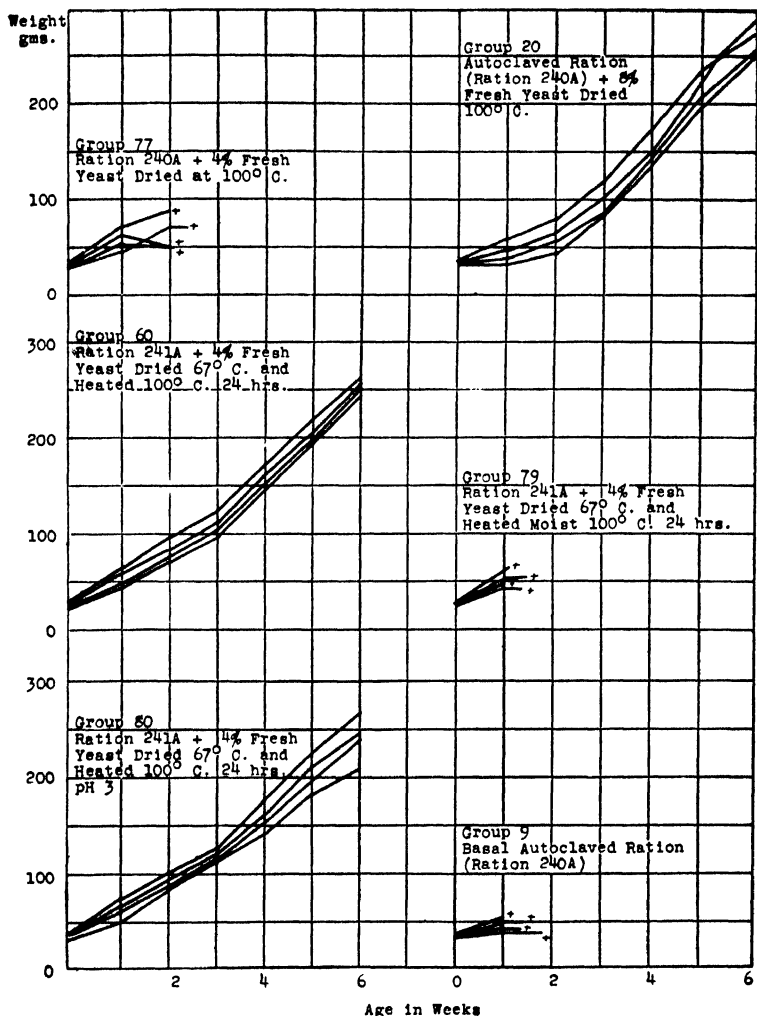


CHART I. Growth records of chicks showing the effect of heat, moisture, and pH on the destruction of vitamin B<sub>1</sub> in yeast.

for 24 hours before being incorporated into Ration 241-A poor growth was obtained both at the 24 and 32 per cent levels. Only

a few of the birds developed severe polyneuritis, which indicates that the destruction was not entirely complete. However, the decided difference in growth demonstrates that the presence or absence of moisture is also an important factor in the destruction of vitamin B<sub>1</sub> in a natural ration.

Since the vitamin B<sub>1</sub> in yeast and in a natural ration is destroyed completely by autoclaving and is destroyed to a large extent by heating *moist* at 100° for 24 hours, and since it is not destroyed when heated *dry* at 100° for 24 hours, it is logical to conclude that the presence of moisture is a more important factor in the destruction than is the increase in temperature. This conclusion substantiates the suggestion made by Sherman and Burton (8) that vitamin B<sub>1</sub> is destroyed by a hydrolysis in which the hydroxyl ions act as a catalyst.

#### *Heat Stability of Vitamin B<sub>2</sub>*

There has been much interest during the past few years in the stability of vitamin B<sub>2</sub> to heat in alkaline media. Some of the workers claim that autoclaving in an alkaline medium destroys the vitamin, while others find no reduced potency under these conditions. Guha (10) has reviewed the work conducted on yeast and yeast extracts and has also shown that the vitamin B<sub>2</sub> in an aqueous solution of commercial liver extract is highly stable while the vitamin B<sub>2</sub> in an extract of fresh liver or fresh yeast is markedly unstable. He suggests that the difference is due to the presence or absence of some protective material in the commercial preparation.

Chick and Roscoe (11) studied the stability of vitamin B<sub>2</sub> at temperatures lower than 120°. They found that yeast heated for 2 hours at 90–100° in a faintly acid solution lost none of its potency, but when heated at pH 8.3 the activity was reduced one-half, and when allowed to remain in alkaline solution for 10 days at room temperature, one-third of the activity was lost.

The results given in the preceding paper clearly demonstrate that vitamin B<sub>2</sub> can be destroyed at temperatures lower than 120°. A few experiments were conducted to determine whether the inactivation of vitamin B<sub>2</sub> is due to heat *per se* or due to a reaction, the rate of which is increased by a higher temperature. Three portions of the basal ration (Ration 240) were heated at 100° for 24, 72, and

144 hours. Groups of four chicks, kept in individual cages, were fed each of these rations. The growth curves, including those for a group of chicks on the basal unheated ration, are given in Chart II. The chicks receiving the basal ration heated for 24 hours grew exceedingly well (Group 12). In fact, the rate of growth was slightly better than that obtained with chicks on the

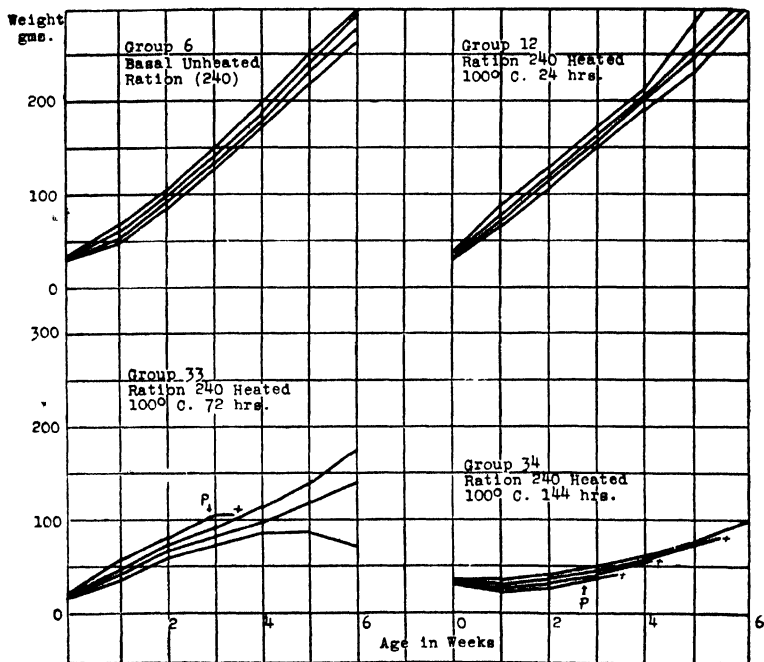


CHART II. Typical growth curves of chicks given Ration 240 (basal unheated ration) heated at 100° for varying periods of time. *P* denotes the approximate time at which the symptoms of pellagra were first observed.

basal ration alone. The application of heat for a short time (100° for 24 hours), therefore, has no deleterious effect on vitamin B<sub>2</sub> but may actually improve the ration for growth. When the heating process is continued for 72 hours considerable destruction of vitamin B<sub>2</sub> takes place (Group 33) and when continued for 144 hours the destruction is complete or nearly so (Group 34).

Knowing that the ration could be rendered free of vitamin B<sub>2</sub> by proper heat treatment, we were interested in determining the

effect of heat on a substance rich in vitamin B<sub>2</sub>, such as yeast. It was first necessary to find approximately the amount of vitamin

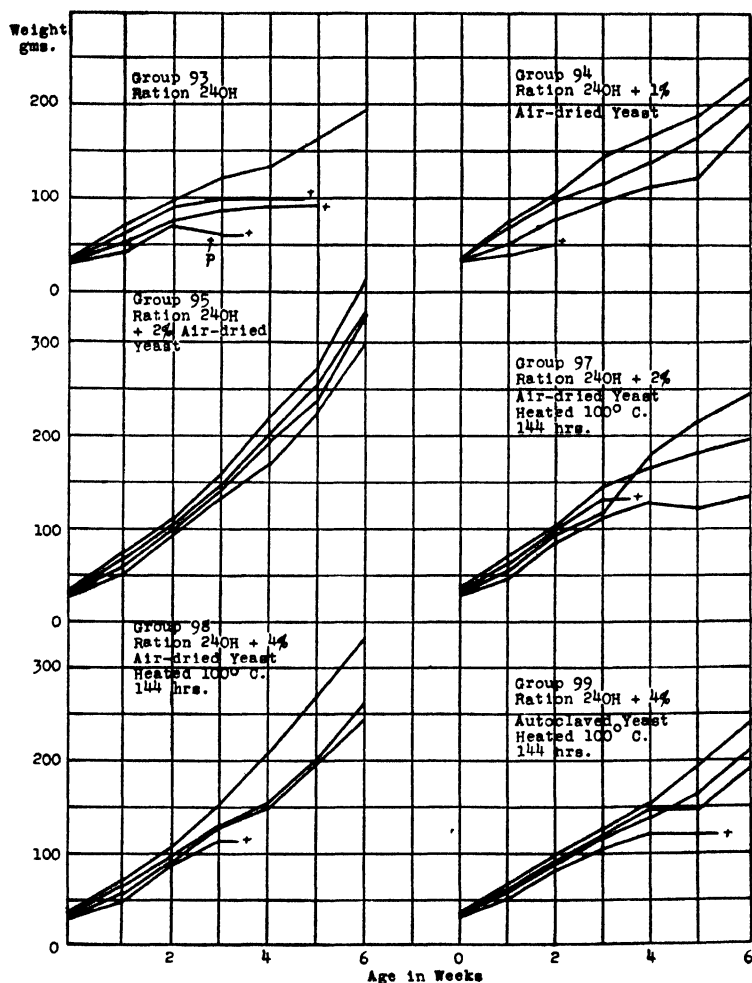


CHART III. Growth records of chicks showing the effect of heat treatment on the destruction of vitamin B<sub>2</sub> in yeast. P denotes the approximate time at which the symptoms of pellagra were first observed.

B<sub>2</sub> present in the yeast used, and this was done by feeding it at levels of 1, 2, and 4 per cent, added to Ration 240-H. It is evident from the growth curves in Chart III for Groups 94 and 95,

that 2 per cent of the yeast used contains sufficient vitamin B<sub>2</sub> for the chick, while 1 per cent is inadequate. When the air-dried yeast was heated at 100° for 144 hours and fed at the 2 per cent level (Group 97), the growth obtained was similar to that of chicks receiving 1 per cent of the untreated yeast. This is an approximate indication that 50 per cent of the vitamin B<sub>2</sub> was destroyed by the heat treatment. When the heated yeast was fed at the 4 per cent level, growth was somewhat improved, but there is still evidence of considerable destruction. When the yeast was first autoclaved and then heated for 144 hours at 100° (Group 99), there was slightly greater destruction than with the long heating alone.

Feeding trials have shown that 50 per cent of the basal Ration 240 is equivalent to 2 per cent of yeast in vitamin B<sub>2</sub> potency. Since yeast contains approximately 25 times more vitamin B<sub>2</sub> than the ration, it is reasonable that only one-half of the vitamin B<sub>2</sub> in the yeast should be destroyed under the conditions we have used. It is possible that with longer periods of heat treatment, a larger part of the vitamin may be destroyed.

The above results suggested that an oxidation process may take place during the heating which is responsible for the destruction. The results obtained from a few preliminary experiments indicate that this is not the case. Considerable destruction was observed when yeast was boiled in a water suspension for 144 hours and when heated in a tube filled with nitrogen. Further work is being continued in order to study more carefully the destruction under these conditions.

#### *Does the Chick Require Vitamin B<sub>3</sub>?*

In 1930, Eddy, Gurin, and Keresztesy (4) concluded that chicks require the unstable vitamin B<sub>3</sub> for growth, even when fully supplied with vitamins B<sub>1</sub> and B<sub>2</sub> and with the other nutrients and vitamins. They found yeast, whole grains, and malt to be good sources of this factor, and that it is much more heat-labile than vitamin B<sub>1</sub>. A temperature of 20° markedly reduced the amount of this factor in yeast if it was submitted to alkali treatment before drying.

During the past 2 years we have fed a number of rations, the constituents of which have been heated to temperatures of 100°

or above. We were greatly surprised to find that chickens made excellent growth on these rations because vitamin B<sub>2</sub> would certainly have been destroyed in our rations if it is as labile as Eddy and coworkers maintain.

The growth curves for chicks grown on three different rations are given in Chart IV. In each case all of the constituents of the

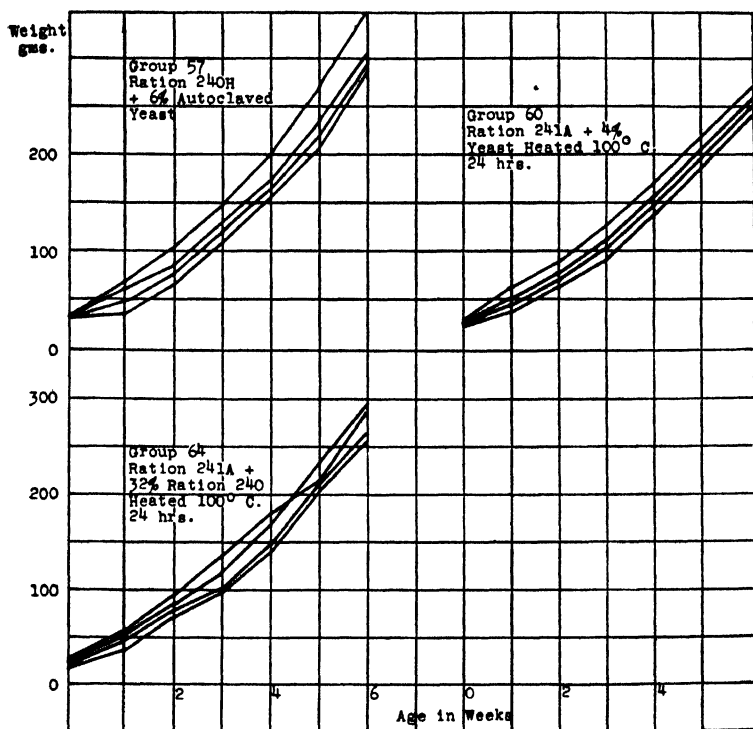


CHART IV. Growth records of chicks grown on rations whose constituents, with the exception of CaCO<sub>3</sub> and cod liver oil, have been heated at 100° or above.

ration except the cod liver oil and CaCO<sub>3</sub> were heated at 100° or above. Group 57 received Ration 240-H plus 6 per cent of autoclaved yeast. Ration 240-H is our basal Ration 240 heated at 100° for 144 hours and is deficient in vitamin B<sub>2</sub>. The autoclaved yeast supplied this deficiency. Group 60 was given Ration

241-A plus 4 per cent of yeast heated dry at 100° for 24 hours. Ration 241-A is our basal Ration 240 autoclaved plus 4 per cent of autoclaved yeast, and is deficient in vitamin B<sub>1</sub>. The heated yeast supplied the lacking vitamin B<sub>1</sub>. Group 64 also received Ration 241-A but in this case the vitamin B<sub>1</sub> was supplied by 32 per cent of Ration 240 heated dry at 100° for 24 hours.

The growth curves in Chart IV show that all of these rations produced normal growth or growth comparable in every way to the unheated ration. The results demonstrate without doubt that chicks do not require vitamin B<sub>3</sub>, or any other factor which is destroyed by temperatures lower than 100°.

#### SUMMARY

1. Vitamin B<sub>1</sub> in yeast and in a natural ration was destroyed completely by autoclaving and inactivated to a large extent by heating moist at 100° for 24 hours, but was not reduced in potency when heated dry at 100° for 24 hours. The rate of destruction of vitamin B<sub>1</sub> in the presence of moisture was decreased with increase in hydrogen ion concentration.

2. The inactivation of vitamin B<sub>2</sub> in a natural ration heated dry at 100° was found to be dependent upon the length of time of heating. No noticeable destruction was observed after 24 hours of heating, but one-half of the potency was lost after 72 hours and the destruction was practically complete after 144 hours of heating. Dried bakers' yeast which contains about 25 times as much vitamin B<sub>2</sub> as the natural ration used lost one-half of its potency after heating dry for 144 hours.

3. Results are presented to show that chickens do not require the heat-labile vitamin B<sub>3</sub> or any other factor which is destroyed by heating at 100° or below for 24 hours.

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# CONFIGURATIONAL RELATIONSHIPS OF METHYL- PHENYL-, METHYLCYCLOHEXYL-, AND METHYLHEXYLCARBINOLS AND OF THEIR HOMOLOGUES

A CORRECTION

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(Received for publication, November 4, 1932)

In the original article<sup>1</sup> an error occurred in calculating the molecular rotations of the acid phthalates of the isopropylcarbinols. The molecular rotations given by Pickard and Kenyon were taken erroneously to be specific and therefore multiplied by 0.01 m. The correct values are as follows:

	CH <sub>3</sub>	C <sub>2</sub> H <sub>5</sub>	C <sub>3</sub> H <sub>7</sub>	C <sub>4</sub> H <sub>9</sub>	C <sub>6</sub> H <sub>11</sub>
	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
Phthalates.....	+98	+10	-22	-36	-44
Carbinols.....	+4.7	-16.7	-27.1	-35.9	-38.2

Thus the shift of the rotation on changing from the carbinols to the phthalates is to the right in the first *three* members and not in the first two, as stated; in the higher members the shift is, as stated in our paper, to the left.

We wish to thank Professor K. Freudenberg for calling our attention to the error.

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).



# THE DETERMINATION OF COLLOID OSMOTIC PRESSURES IN SMALL QUANTITIES OF FLUID

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(Received for publication, October 10, 1932)

At the suggestion of Professor Krogh, the possibility was investigated of adapting the newer osmometer<sup>1</sup> of Krogh and Nakazawa (1) to use with small volumes of colloid solutions. We first tried to reduce the volume of the solution by replacing part of it with mineral oil. The osmometer was assembled in the usual way and a layer of about 0.3 cc. of the solution to be tested, introduced. Then enough light mineral oil was added to fill the chamber and form the column in the capillary tube. This method, however, gave irregular results and was soon abandoned. The oil attacked the rubber parts of the apparatus and the character of the irregularities suggested that surface effects at the interface might be causing changes in the solution.

Osmometers of 0.3 cc. capacity were then used. No changes were made in the technique previously described (1, 2). The slight changes made in the apparatus are given below. Horse serum, gum arabic solution, and the sera of some fresh and sea water fishes were used. Determinations were made both with the small capacity osmometers and with those of 2.0 cc. capacity. Readings were made at hourly intervals. The final values reached agreed very closely and the time required to reach equilibrium was about one-third less with the small volume apparatus. In the case of the gum solution, nine determinations with the small volumes gave an average final reading of 253 mm. of water and

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<sup>1</sup> For details of the use of the apparatus, see the paper of Krogh and Nakazawa (1) and that of Turner (2).

all had reached equilibrium in 3.6 hours. Four determinations on the same solution with large volumes gave a final average value of 248 mm. of water and all were in equilibrium in 5.0 hours. In comparing the two techniques it should be pointed out that the relative time only can be given, since the actual time varies greatly

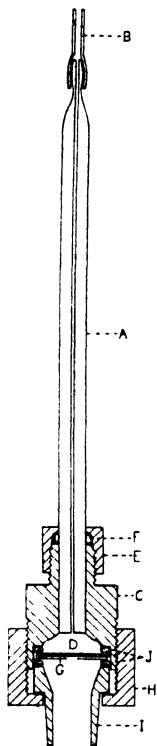


FIG. 1. Diagram of osmometer; reprinted from Turner (2). *A*, capillary; *B*, rubber tube; *C*, hard rubber section; *D*, chamber for liquid to be tested; *E*, metal nut; *F*, rubber washer; *G*, perforated silver disc; *H*, hard rubber screw; *I*, hard rubber conical section; *J*, rubber washers.

with the permeability of the membrane and particularly with the height of the pressure finally attained. Equilibrium is reached very much more quickly when the colloid osmotic pressure of the solution under investigation is low. When it is as low as 100 mm. of water, equilibrium is reached in from 1.5 to 2.0 hours with the small volume apparatus.

The following slight changes in the apparatus and points in technique are important (Fig. 1): (1) The capacity of the chamber, *D*, should be not more than 0.3 cc. (2) The inside washer, *J*, should be made from heavy rubber dam. Using rubber dam reduces the capacity of the chamber, but the lighter rubber dam has a tendency to curl after use and is difficult to handle. (3) The glass capillaries should fit closely into the neck of the part *C* so that the space left will require very little solution to fill it. (4) The depression for receiving the membrane in *C* should be quite shallow (2 mm.). (5) The capillary tube may be pushed down nearly to the membrane, further reducing the capacity of the apparatus. (6) A preliminary reading should be made in every case about 1 hour before the final reading is made, in order that the pressure imposed on the system will be close to the colloid osmotic pressure of the solution under investigation.

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## ON THE PURIFICATION AND CONSTITUTION OF THEELOL

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(Received for publication, October 29, 1932)

During the last 2 years some difficulties in the preparation of theelol have been encountered and overcome. The new procedures which are described in this paper are important in that they have enabled us to obtain crystalline theelol from samples that gave only a brown tar by the method already published (Doisy and Thayer, 1931). The earlier procedure is frequently satisfactory but the improvements are distinctly advantageous in the treatment of grossly impure products.

In the event that one obtains a tar when the alkaline solution of Step 7 (Doisy and Thayer, 1931, p. 642) is acidified, we have found it advantageous to add sodium hydroxide until solution is effected. The alkaline solution is then transferred to a separatory funnel, extracted with butyl alcohol, and the butyl alcohol distilled. The residue which contains most of the theelol is dissolved in a small volume, 50 to 100 cc., of butyl alcohol, 2 volumes of petroleum ether are added, and the mixture is washed two or three times with a small volume of aqueous sodium carbonate. The theelol is then extracted from the butyl alcohol-petroleum ether solution by shaking repeatedly with dilute sodium hydroxide. Acidification of the alkaline solution will precipitate the theelol which should then be recrystallized from ethyl alcohol.

An alternative procedure which in many instances has proved satisfactory is based upon the insolubility of the sodium (or potassium) salt of theelol. The alkaline solution (of Step 7 of the original procedure) is merely concentrated on a boiling water bath until the salt precipitates. After cooling, the precipitate is

removed by centrifugation, dissolved in dilute sodium hydroxide, stirred with norit, filtered, and precipitated with acetic acid. Digestion on the steam bath flocculates the precipitate so that it filters readily. Precipitation with hydrochloric acid is to be avoided since impurities upon warming yield a deep blue color which can be removed from theelol with difficulty.

The prolonged heating with sodium hydroxide does not alter theelol. Samples so treated have the same melting point, optical rotation, and physiological activity as our early preparations. Furthermore, 63 mg. of theelol (m.p. 272.5°, uncorrected) were dissolved in 20 cc. of 10 per cent sodium hydroxide solution and heated in a sealed tube at 100° for 52 hours. The theelol was precipitated from the pale yellow solution by addition of hydrochloric acid and crystallized from dilute alcohol. Recrystallization gave 37 mg. of typical theelol crystals which melted at 273°, uncorrected. The optical rotation was unchanged and the assay was 1500 rat units per mg.

The report of Butenandt that his early preparations contained the ketohydroxy compound (our theelin) as an impurity has led us to examine some of our products by the procedure described by him. In no instance have we obtained any semicarbazone, nor have we found any diminution of the potency of the preparations. Two examples are given in the following paragraphs.

*Purification with Semicarbazide Hydrochloride. Theelol Preparation M-222; Assay 1500 Rat Units per Mg.*—100 mg. of theelol were dissolved in 20 cc. of 95 per cent ethyl alcohol. An aqueous solution of 80 mg. of semicarbazide hydrochloride and 80 mg. of sodium acetate was added and the mixture allowed to stand for 7 days at room temperature. Since no precipitate had formed, the solution was evaporated to dryness and the residue dissolved in methyl alcohol. A small volume of ethyl acetate was added. No crystals of theelin semicarbazone could be obtained from this solution. After evaporating the solution to dryness, the residue was dissolved in ethyl alcohol and the theelol was precipitated by adding 3 volumes of water. It was recrystallized from dilute alcohol. M.p. 281°; C 75.19 per cent, H 8.43 per cent.

No nitrogen was obtained by the micro-Dumas method in 9.26 mg.; assay, 1500 rat units per mg. To another 100 mg. of Preparation M-222, 2 mg. of theelin were added and the mixture was

treated with semicarbazide hydrochloride and sodium acetate in an identical manner. Theelin was isolated as the semicarbazone; m.p. 253° with decomposition.

*Purification with Hydroxylamine. Theelol Preparation M-222; Assay 1500 Rat Units per Mg.*—72 mg. of theelol were refluxed with hydroxylamine, 2 volumes of water were added, and the precipitate was filtered off. It was dissolved in ethyl alcohol and the precipitation with water repeated. M.p. 272.5° uncorrected; no nitrogen was obtained by the micro-Dumas method in 5.410 mg.; assay, 1500 rat units per mg. The mother liquors were combined, heated, and water was added to produce a turbidity. The tube was allowed to cool and the crystals removed by filtration. They were dissolved in ethyl alcohol and the crystallization repeated twice. No nitrogen was obtained by the micro-Dumas method in 6.476 mg.

In spite of the failure to find any theelin in our theelol by procedures designed to detect the former, it is a fact that the melting point reported by Butenandt is somewhat higher than our uncorrected melting points. Since this may be due to differences in our procedures, it may be well to state just how we take melting points.

We use the open beaker method and heat at such a rate that the temperature rises steadily at a rate of 2° per minute. The temperature at which the crystals run together to form a meniscus in the bottom of the tube is taken as the melting point (see deJong, Kober, and Laqueur, 1931). Our uncorrected values were obtained with a Bureau of Standards 360° thermometer with a stem exposure beginning at 0°. Wherever temperatures are not indicated as uncorrected in this paper they were obtained with a short stem thermometer requiring no correction for stem exposure. With this thermometer, under the conditions given, the melting point for theelol is 282–283°.

In the course of our investigations on the constitution of theelol we have obtained several new substances.<sup>1</sup> These same com-

<sup>1</sup> The preparation of these compounds (except desoxotheelin) was reported by one of us (E. A. D.) during a symposium on sex hormones at a meeting of the Section on Biochemistry and Physiology of the British Medical Association in London on August 28, 1932. Dr. Marrian who also discussed the degradation of the trihydroxy compound (theelol) at that meeting has recently published his experimental data (Marrian and Haslewood, 1932).

pounds have been recently described by Marrian and Haslewood (1932) but inasmuch as our experiments differ somewhat from theirs and our melting points are significantly higher we feel justified in presenting a brief description of this work.

In agreement with the work of Marrian and Haslewood we have likewise found that alkali fusion of theelol results in the conversion of both aliphatic hydroxyl groups to carboxyl groups, giving the phenolic dibasic acid,  $C_{18}H_{22}O_6$ , which result indicates that the hydroxyl groups are on adjacent carbon atoms and that a ring has been opened between them. On this basis Butenandt's (1931) conversion of theelol to theelin by heating with potassium acid sulfate is easily understandable.

We also found that this phenolic dibasic acid,  $C_{18}H_{22}O_6$ , readily forms a methyl ether,  $C_{19}H_{24}O_6$ , and that this latter acid on treatment with acetic anhydride and subsequent distillation forms an anhydride and not a ketone. That no trace of ketone is formed is shown by the complete solubility of the sublimate in warm dilute alkali. While this evidence does not constitute an entirely secure basis for speculation, we are inclined to agree with Marrian and Haslewood that the most probable indication is that the ring bearing the two aliphatic hydroxyl groups is 5-membered rather than 6-membered.

An investigation of the bromine compounds isolated from the reaction mixtures after the determination of iodine numbers has led Marrian and Haslewood (1932) to the conclusion that theelol and theelin contain no fourth double linkage, the apparent addition of a molecule of halogen being actually a substitution. Work by Butenandt, Störmer, and Westphal (1932) on the catalytic reduction of theelol leads to the same conclusion. These latter workers hydrogenated theelol with the aid of a platinum catalyst and found that only 6 atoms of hydrogen were taken up; that is, just sufficient to reduce the aromatic nucleus. We have found that reduction of theelol with a nickel catalyst yields a hexahydro derivative apparently identical with that of Butenandt and coworkers. The weight of evidence thus appears to be against the presence of an ethylenic linkage in the theelol molecule.

A Clemmensen reduction product of theelin has been described by Butenandt, Störmer, and Westphal (1932) and also by Marrian and Haslewood (1932). Butenandt *et al.* assigned the formula,

$C_{18}H_{26}O$ , to their compound which melted at  $129^{\circ}$  and was insoluble in alkali. The compound obtained by Marrian and Haslewood melted at  $133\text{--}134^{\circ}$ , gave an analysis corresponding to the formula,  $C_{18}H_{24}O$ , and was stated to be insoluble, or nearly so, in alkali. We have prepared desoxotheelin by the Clemmensen reduction of theelin following the procedure described by Marrian and Haslewood, and our results are in substantial agreement with theirs. The calculated values for  $C_{18}H_{24}O$  are: C 84.33, H 9.44 per cent; and for  $C_{18}H_{26}O$ : C 83.67, H 10.15 per cent. Analysis of our compound gave C 84.56, H 9.46 per cent., which figures agree better with the formula,  $C_{18}H_{24}O$ , assigned to the compound by Marrian and Haslewood. The melting point was  $132\text{--}133.5^{\circ}$ , uncorrected. Upon boiling with 0.1 N aqueous sodium hydroxide the substance dissolved slowly but sparingly. Acidification of the clear solution caused precipitation of the desoxotheelin.

*Formation of Phenolic Dibasic Acid,  $C_{18}H_{22}O_6$ , from Theelol*—500 mg. of theelol were stirred into a melt of 10 gm. of potassium hydroxide and 1.5 cc. of water in a nickel crucible held at  $275^{\circ}$  in a bath of fused sodium and potassium nitrates. Heating at this temperature was continued for 1 hour with occasional stirring. After the melt had cooled it was dissolved in water and filtered from a small amount of nickel oxide. Saturation of the solution with carbon dioxide resulted in the precipitation of about 50 mg. of theelol which was removed by filtration. The filtrate was acidified with sulfuric acid, cooled thoroughly, and the precipitated acid filtered off, washed with water, and dissolved in 95 per cent alcohol. The light brown solution was treated with norit and the acid subsequently crystallized from dilute alcohol. We were unable to purify this substance satisfactorily by crystallization. Our product, like that of Marrian and Haslewood, melted over a range of  $6\text{--}7^{\circ}$ . A very effective means of purification was found, however, in the precipitation of the potassium salt from absolute alcohol. The material was dissolved in absolute alcohol and treated with a solution of potassium hydroxide in absolute alcohol. The granular white potassium salt was filtered off, washed with absolute alcohol, dissolved in water, and the acid precipitated by means of hydrochloric acid. The yield of acid thus obtained was 330 mg. By repeated crystallization from dilute alcohol, with the use of norit when necessary, the acid was obtained in the form

of beautiful large white needle-like plates which contained 1 molecule of water of hydration which was removed by heating in a vacuum at 135° over phosphorus pentoxide, or by standing over sulfuric acid. The anhydrous acid melted at 210–211° (207–208°, uncorrected).

$C_{18}H_{22}O_5$ .	Calculated.	C 67.88, H 6.97, mol. wt. 318
	Found.	" 67.35, 67.55, H 7.03, 6.88, mol. wt. 318, 320 (Rast)

$C_{18}H_{22}O_5 \cdot H_2O$ .	Calculated.	$H_2O$ 5.36
	Found.	" 4.73, 4.88

The sulfuric acid filtrate from the phenolic dibasic acid was distilled in an all-glass distilling apparatus and the distillate titrated with standard alkali. Approximately 1 cc. of 0.1 N sodium hydroxide was required. By evaporation of this solution on the water bath a minute amount of sodium salt was obtained which, when moistened with sulfuric acid, evolved a volatile fatty acid. The amount was too small for identification but the odor seemed to be that of acetic acid. It is probably formed as a result of a more extensive degradation of a small part of the theelol during the fusion, and is perhaps somewhat insecure evidence in favor of the presence of a methyl side chain somewhere in the molecule.

*Methoxy Dibasic Acid*—575 mg. of acid,  $C_{18}H_{22}O_5$ , were dissolved in 15 cc. of methyl alcohol and 2 cc. of redistilled dimethyl sulfate added. 15 cc. of 25 per cent potassium hydroxide were added and the mixture was heated in an oil bath at 110°, under a reflux condenser for  $\frac{1}{2}$  hour. Then 1 cc. of dimethyl sulfate and 10 cc. of potassium hydroxide were added and the heating was continued for 2 hours. A small amount of ester separated as an oil and this was saponified by further heating with additional alkali. The cold solution was acidified with hydrochloric acid; whereupon, the methoxy acid separated as a white precipitate. This was filtered off, dissolved in methyl alcohol, and treated with norit to remove a small amount of color. After two recrystallizations from dilute methyl alcohol the yield of white crystalline product was 538 mg. Further recrystallization from the same solvent gave a product which melted at 200–201° (196–197°, uncorrected).

$C_{18}H_{24}O_5$ .	Calculated.	C 68.63, H 7.28, $CH_3O$ 9.33, mol. wt. 332
	Found.	" 68.49, 68.59, H 7.1, 7.1, $CH_3O$ 9.68, 9.70, 9.33, mol. wt., calculated from $CH_3O$ value, 324

*Methoxy Acid Anhydride*,  $C_{19}H_{22}O_4$ —50 mg. of methoxy acid,  $C_{19}H_{24}O_5$ , were refluxed with 1 cc. of pure acetic anhydride in an oil bath held at  $175^\circ$  for 6 hours. The acetic anhydride was then removed by distillation leaving a light brown crystalline residue. This was heated slowly in an oil bath to  $250^\circ$  and was then distilled at that temperature and 0.018 mm. pressure. The pale yellow crystalline distillate was dissolved in a mixture of glacial acetic acid and acetic anhydride and filtered through a sintered glass filter. The solution was concentrated in an oil bath to about 1 cc. and a drop or two of acetic anhydride added. On standing in the refrigerator the solution deposited well formed white needles. This product was filtered off and washed with 2 or 3 drops of acetic anhydride. After drying at 15 mm. and  $110^\circ$  the product sintered slightly at  $171^\circ$  and melted at  $179^\circ$  ( $176^\circ$ , uncorrected).

$C_{19}H_{22}O_4$ .	Calculated.	C 72.56, H 7.06, $CH_3O$ 9.87
	Found.	" 72.59, 72.18, H 7.24, 7.42, $CH_3O$ 9.81

*Catalytic Reduction of Theelol*—500 mg. of theelol were dissolved in 70 cc. of absolute alcohol and 2 gm. of a nickel catalyst added. This was then shaken with hydrogen at 150 atmospheres and  $175^\circ$  for 1 hour.<sup>2</sup> The catalyst was removed by filtration and the filtrate concentrated on the water bath to 5.0 cc. The addition of 4 or 5 volumes of 2 per cent potassium hydroxide solution precipitated the reduction product as a pale yellow viscous oil. This was separated by decantation, dissolved in 95 per cent alcohol, and the pale yellow solution decolorized by treatment with norit. Attempts were made to crystallize the product from a wide variety of solvents but it invariably separated as an oil. By means of cold acetone it was separated into two fractions, one of which was considerably more soluble than the other. Upon evaporation of the acetone the more soluble fraction was obtained as a glassy, semi-solid mass which we have so far been unable to crystallize. The less soluble fraction was repeatedly crystallized from dilute alcohol and from ethyl acetate. It sintered somewhat at  $240^\circ$  and melted at  $268^\circ$  ( $260^\circ$ , uncorrected). The hexahydro compound ob-

<sup>2</sup> We wish to express our thanks to Dr. L. W. Covert and Professor Homer Adkins of the University of Wisconsin who carried out this reduction with their nickel catalyst No. 24 Cv.

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tained by Butenandt and coworkers melted at 255–256°, uncorrected.

$C_{18}H_{30}O_3$ .	Calculated.	C 73.42, H 10.28
	Found.	" 73.69, 73.66, 73.61, H 10.38, 10.28, 10.23

### SUMMARY

1. An improved method for the purification of theelol has been described which is based on the insolubility of the alkali metal salts in solutions of the hydroxides of these metals. Prolonged treatment of theelol with strong alkali does not alter its physiological activity, optical rotation, or melting point.

2. Treatment with ketone reagents failed to reveal the presence of any theelin in our routine preparations of purified theelol.

3. Alkali fusion of theelol results in the formation of a phenolic dibasic acid,  $C_{18}H_{22}O_5$ . The methyl ether of this acid on treatment with acetic anhydride and distillation in a vacuum yields an acid anhydride,  $C_{19}H_{22}O_4$ . No ketone is formed. The most probable conclusion to be drawn from these facts is that the theelol molecule contains a 5-membered carbon ring bearing two secondary hydroxyl groups on adjacent carbon atoms.

4. Catalytic reduction of theelol by means of a nickel catalyst yields a hexahydro derivative which is insoluble in alkali.

5. Desoxotheelin was prepared by the Clemmensen reduction of theelin and found to have the formula,  $C_{18}H_{24}O$ . The substance is slightly soluble in dilute aqueous alkali.

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**TRANSFERENCE AND CONDUCTIVITY STUDIES ON  
SOLUTIONS OF CERTAIN PROTEINS AND AMINO  
ACIDS WITH SPECIAL REFERENCE TO THE  
FORMATION OF COMPLEX IONS  
BETWEEN THE ALKALINE  
EARTH ELEMENTS AND  
CERTAIN PROTEINS\***

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The present investigation is a continuation of the systematic studies which have been carried out in this laboratory on the mode of union between proteins and amino acids with the inorganic elements. Previous reports have dealt with the compounds of proteins with the alkali metals (1), the alkaline earth elements (2), iron (3), and manganous ion (4).

By means of electrical transference measurements, Greenberg and Schmidt (1) were able to show that within the limits of error, which may be assumed to be about 10 per cent, the current is carried by the cations of the alkali metal and the casein anions. It was found further that the value of the mobility of the casein anions determined from transference experiments was about the same as that which they determined from conductivity measurements (5). On the other hand, solutions of casein and of the serum proteins, when combined with the alkaline earth elements, yielded abnormal values for the transference numbers, indicating that a considerable portion of the cations was bound to the protein to

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yield negatively charged complex ions of protein and alkaline earth element. The conductivity experiments of Robertson (6) and of Adolf (7) point in the same direction. Kirk and Schmidt (8) measured the sodium and the barium ion activities in casein solutions. Their conclusions support the previously mentioned results of Greenberg and Schmidt. The fact that only a portion of the serum calcium is diffusible probably indicates that it is held in complex combination with the serum proteins.

Greenberg and Schmidt (9) explained the formation of the complex compound of casein and alkali earth elements by means of the following equation.

$$M_a\text{casein} = xM^{++} + M_{(a-x)}\text{casein}^{-2x} \quad (1)$$

where  $M$  stands for the alkali earth element,  $a$ , the concentration, and  $x$ , the fraction of casein dissociated. In the case of the iron complexes, Smythe and Schmidt were able to show that certain groups in the protein molecule were responsible for this phenomenon. They offered an explanation, based on the residual charge of atoms, to account for the manner in which the iron was united.

In the case of the alkaline earth elements, no explanation to account for their behavior in forming complex ions is available. It is the purpose of this paper to throw further light upon this subject. Since solutions of the alkaline earth hydroxides are strongly basic, it is to be expected that they would neutralize the acidic groups which are present in the protein molecule (10). The acidic groups of casein are: the carboxyl groups of aspartic, glutamic, and hydroxyglutamic acids, phosphoric acid, and the hydroxyphenyl group of tyrosine. It was therefore to these groups that we have looked for an explanation of the seat of formation of the complex ions. Our experiments have incidentally thrown considerable light upon the behavior of solutions of certain amino acids and of casein.

#### EXPERIMENTAL

The plan of the experiments was to determine the transference numbers and the conductivity of solutions of both casein and of dephosphorized casein and of solutions of aspartic and of glutamic acids. Similar work was not carried out on solutions of tyrosine since over the range of acidities at which transference experiments

can be carried out, tyrosine does not combine appreciably with either the alkali metals or the alkaline earth elements.

Casein was prepared from milk according to the procedure of Van Slyke and Baker (11) as modified by Greenberg and Schmidt (12). Dephosphorized casein was prepared from the casein mentioned above and from a purified commercial preparation according to the procedure which has been described by Rimington and Kay (13). The analyses of these products were as follows:

	Casein	Dephosphor- ized casein
	<i>per cent</i>	<i>per cent</i>
Ash content.....	0.21	0.0
Moisture lost at 105°.....	2.8	3.4
Phosphorus.....	0.8	0.0
Nitrogen.....	14.6	13.2

The difference in nitrogen content between casein and dephosphorized casein may be due to either one of the following possibilities, or to both: (a) Dephosphorized casein is a complex split-product of casein; or (b) a part or all of the amide nitrogen of casein is lost due to the action of the alkali (14). Rimington (15) states that the essential difference between casein and dephosphorized casein lies in the content of phosphorus.

The aspartic acid was an Eastman Kodak Company product. Glutamic acid was prepared from ajinomoto according to the technique which has been described by Schmidt and Foster (16), care being taken to prevent the formation of pyrrolidonecarboxylic acid. The glycine was a Pfanstiehl Chemical Company product. All of the amino acids were recrystallized at least three times from conductivity water and dried over  $P_2O_5$  before use.

The sodium hydroxide solution was prepared by electrolysis. Barium hydroxide was recrystallized from a hot supersaturated solution and a solution was made by the use of conductivity water. Calcium hydroxide solution was prepared by dissolving calcium oxide in conductivity water. All of these solutions were standardized against pure oxalic acid.

Conductivity water was prepared by redistilling laboratory-distilled water to which a small quantity of sodium hydroxide and potassium permanganate had been added. It was stored in Jena

glass bottles. The average conductivity was about  $1.5 \times 10^{-6}$  mhos.

The experimental procedure for the determination of transference numbers of the proteins was the same as that which has been described by Greenberg and Schmidt (12). All determinations were carried out in duplicate. The transference cells were of Pyrex glass, with 10 mm. bore stop-cocks between the middle and the end compartments. The capacity of each compartment was about 40 cc. A platinum wire was used as anode and a lead wire covered with lead peroxide served as cathode. About 2 to 10 milliamperes of a 220 volt current were passed through the protein solutions. The determinations were carried out in an air bath at 25°.

The analyses of casein and of dephosphorized casein were made by drying 10 gm. portions in porcelain crucibles at 105° to constant weight. The weight of cations in the solution was subtracted from the total. The method of estimating the cations consisted in titrating with trichloroacetic acid to the isoelectric point of casein. The protein acts as its own indicator. The calculation of the transference numbers was the same as that which has been described by Greenberg and Schmidt (12).

The method which was used for the determination of the transference numbers of the amino acids deserves some comment. The cell was the same as that which was employed for estimating transference numbers in casein solutions. It was, however, inverted. Both electrodes were made of platinum gauze, each of which was welded to a platinum wire. The wires passed through rubber stoppers. A small hole in each stopper permitted equalization of the internal with the external pressure. Both electrodes were placed in the top of the side tubes of the glass vessel and the bottom was closed by means of rubber stoppers.

Preliminary experiments showed that the acidity of both the anode and the cathode compartment varied considerably, which seriously interfered with the determination of the transference numbers. The difficulty was overcome by covering the platinum cathode with crystalline aspartic or glutamic acid, the whole being enclosed in a bag made of collodion or of filter paper. Since these amino acids are fairly insoluble, the acidity is not materially affected. They serve, however, to prevent change of acidity by

combining with the excess of barium or calcium ions which migrate to the cathode during the course of the experiment.

The platinum anode was covered with either recrystallized barium or calcium carbonate. The anode was likewise placed either in a collodion or a paper bag. Both of these compounds are sufficiently insoluble to be without influence on the acidity of the solution. They served to neutralize the acid which migrated to the anode. The carbon dioxide which was liberated escaped to the outer atmosphere, and since the electrode was placed in the top of the compartment, stirring due to escape of gas was eliminated. The use of the above technique prevented any material change in the acidity of the amino acid solutions and reproducible results were easily obtained. While the above technique served in the case of the calcium or barium salts of aspartic and glutamic acids, a further modification was found necessary in the case of the sodium salts of these amino acids. In the latter instance, metallic cadmium was used for the anode. The *paH* was thus kept constant but the difficulty arose in that, besides sodium ions, cadmium ions also migrated to the cathode. It was, therefore, only possible to calculate the transference numbers of the anions.

The amino acid solution was made by placing a weighed amount of the amino acid in an Erlenmeyer flask which was fitted with a 2-hole stopper. A soda-lime tube was introduced into one hole and a burette which delivered either base or conductivity water was introduced into the other hole. In this way, contamination with carbon dioxide was avoided. Solution was effected by warming to 60°. The amino acid solutions were prepared on the basis of gm. equivalents per 1000 gm. of water.

Two silver coulometers were used in the transference experiments, one at the anode and the other at the cathode end of the apparatus. Agreement between the two coulometers is evidence that there has been no leakage of current. An iodine coulometer was also used at times. This checked very well with the silver coulometer. The amino acid experiments were likewise carried out in an air bath at 25°. In determining the transference numbers of amino acids, two 24 volt dry cells connected in series were used as the source of the current. It was not found desirable to use higher voltages due to the "pumping effect."

The nitrogen estimations were carried out by the Kjeldahl

method. Barium was estimated as the sulfate and calcium was determined according to the method of Fiske and Adams (17). All analyses were carried out in duplicate. Estimation of both amino and total nitrogen indicated that no appreciable decomposition of the amino acids took place at the electrodes.

The conductivity estimations were carried out according to the well known Kohlrausch method. A General Radio Company oscillator, which delivered 1000 cycles per second, was used as the current source, and the measurements were made with the aid of a Leeds and Northrup slide-wire bridge and telephone. The platinum electrodes were frequently coated with platinum black. The cells which were used for measuring the conductivity of the casein solutions were of the vertical type. The cell constants were 0.2147 and 0.1538, respectively. The cells which were used for measuring the conductivities of the amino acids and their salts were of the horizontal type. The cell constants were 1.2735, 0.4251, 0.2147, 0.1538, and 0.01428, respectively. Determination of the cell constants was carried out according to the technique of Parker and Parker (18). Unless otherwise noted, the technique described by Greenberg and Schmidt (5), and later improved by Gahl and Greves (19), for measuring conductivity was followed. The cell was maintained at the desired temperature by immersion in an oil bath, the temperature of which was kept constant within  $\pm 0.01^\circ$ . Readings were made at 30 minute intervals until constant values were obtained. The *paH* of the solutions was determined with the aid of the quinhydrone electrode. The specific conductivity of water (and when necessary the specific conductivity of hydrogen ion) was subtracted from that of the solution.

For purposes of conductivity measurements, 2.5 to 3.0 per cent stock solutions of casein were made. Suitable dilutions of the stock solution were subsequently made. The *paH* of the casein solutions was about 6.5.

The data for the casein and dephosphorized casein transference experiments are given in Table I, and similar data for the two amino acids are given in Table II. In the case of casein and of dephosphorized casein, transference numbers were calculated for the anode portion only. While the *paH* in the cathode compartment remained constant, the presence of  $\text{Pb-PbO}_2$  led to a complex reaction which affected the results.

TABLE I

*Transference Numbers of Sodium and Calcium Caseinate and of Sodium and Calcium Dephosphorized Caseinate at 30°*

	0.1 N silver deposited in coulometer	Q	B	QB = K	T <sub>casein</sub>	T <sub>Na</sub>	T <sub>casein</sub> + T <sub>Na</sub>	i
	cc.							per cent
Sodium caseinate	1.70	1.37	6.52	8.95	0.499	0.542	1.041	
	4.50	1.09	8.55	9.33	0.462	0.534	0.996	
	4.60	1.02	9.05	9.23	0.454	0.548	1.002	
		1.73	5.6	9.68	0.453	0.561	1.014	
*		1.34	7.0	9.40	0.455	0.540	0.995	
*		1.04	8.25	8.60	0.430			
Sodium dephosphorized caseinate	1.70	1.39	6.47	9.00	0.389	0.595	0.984	
	1.35	1.17	6.50	7.61	0.428	0.586	1.014	
	1.35	1.15	6.83	7.89	0.383	0.575	0.958	
	2.05	1.24	7.30	9.05	0.388	0.700	1.088	
	2.75	1.12	7.90	8.85	0.396	0.625	1.021	
Calcium caseinate	1.70	0.945	8.85	8.36	0.424	0.593	1.017	
	1.90	0.411	12.23	5.03	0.953	0.331	1.284	48.7
	1.95	0.331	12.95	4.28	1.185	0.338	1.523	49.3
	1.60	0.300	13.20	3.96	0.968	0.194	1.162	40.5
	1.95	0.2195	14.70	3.23	1.295	0.343	1.638	49.7
	1.90	0.196	14.75	2.90	1.070	0.278	1.348	45.4
	1.60	0.197	15.40	3.02	1.200	0.199	1.399	40.8
Calcium dephosphorized caseinate	1.40	0.962	7.49	7.20	0.542	0.479	1.021	62.8
	1.90	0.972	8.48	8.24	0.564	0.533	1.097	69.8
	1.55	0.723	9.54	6.90	0.608	0.416	1.024	55.9
	1.60	0.614	11.80	7.24	0.676	0.431	1.107	57.9
	1.75	0.666	10.85	7.22	0.580	0.408	0.988	55.1
	1.70	0.614	12.35	7.57	0.686	0.395	1.081	54.0
	1.43	0.614	12.35	7.58	0.657	0.441	1.098	58.2
	2.00	0.481	13.43	6.47	0.630	0.430	1.060	57.3
	1.95	0.531	14.10	7.46	0.700	0.422	1.122	56.6

B = cc. of 0.1 N alkali per gm. of casein; Q = electrochemical equivalent per millifaraday; i = fraction of metallic element which exists as ions; T = transport number.

\* These data are taken from Greenberg and Schmidt (12).

The transference number of the anion is given by the equation

$$T_{\text{anion}} = \frac{\Lambda_{\text{anion}}^0}{\Lambda_{\text{anion}}^0 + \Lambda_{\text{cation}}^0} \quad (2)$$

in which  $\Lambda^0$  is the mobility or equivalent conductivity at infinite

TABLE II  
*Transference Numbers of Amino Acid Solutions at 25°*

	0.1 N silver deposited in coulometer	0.1 N amino acid transferred to anode	0.1 N cation transferred to cathode	$T_{\text{amino acid}}$	$T_{\text{cation}}$	$T_{\text{amino acid}} + T_{\text{cation}}$
	cc	cc	cc			
Monosodium aspartate	9 80	3 44		0 351		
	15 52	5 42		0 349		
	15 52	5 43		0 350		
	16 50	5 90		0 357		
	15 45	5 46		0 354		
	14 20	4 99		0 352		
Monosodium glutamate	9 72	3 10		0 319		
	9 72	3 13		0 322		
	12 05	3 78		0 313		
	15 08	4 90		0 325		
	15 65	5 09		0 324		
	16 95	5 34		0 315		
Barium di- aspartate	15 55	4 79	10 50	0 307	0 676	0 983
	19 40	5 75	13 80	0 297	0 690	0 987
	21 17	6 45	14 70	0 305	0 695	1 000
	15 25	4 55	10 75	0 298	0 705	1 003
	19 60	5 90	13 80	0 301	0 704	1 005
	19 65	5 92		0 301		
Barium di- glutamate	17 30	4 83	12 79	0 279	0 739	1 018
	14 71	4 25	10 40	0 289	0 709	0 998
	20 17	5 60	14 20	0 278	0 710	0 988
	17 30	4 77	12 50	0 275	0 725	1 000
	9 31	2 59	6 67	0 277	0 717	0 994
	25 55	7 12	18 30	0 279	0 718	0 997
Calcium di- aspartate	14 71	4 34	10 45	0 294	0 711	1 005
	16 89	5 68	11 15	0 336	0 661	0 997
	15 48	4 98	10 60	0 322	0 685	1 007
	17 35	5 53	11 80	0 319	0 678	0 997
	15 33	5 06	10 45	0 329	0 682	1 011
	15 28	4 75	10 52	0 311	0 691	1 002
Calcium di- glutamate	16 69	5 10	11 40	0 306	0 683	0 989
	14 68	4 71	10 22	0 321	0 698	1 019
	16 60	5 00	11 40	0 305	0 692	0 997
	12 79	3 95	9 00	0 306	0 702	1 008
	11 68	3 59	7 94	0 307	0 681	0 988
	11 68	3 39	8 18	0 291	0 701	0 992
	12 79	3 95	9 00	0 306	0 702	1 008
	11 68	3 43	8 41	0 294	0 720	1 014
	9 62	2 89	6 75	0 300	0 702	1 002
	13 26	3 93	9 40	0 296	0 708	1 004

In determining the transference numbers of the salts of amino acids, the

TABLE III

*Comparison of Mobilities of Anions from Data of Conductivities and of Transference Numbers\**

Compound	$\Lambda^\circ_{\text{com-}}\text{pound}$	$\Lambda^\circ_{\text{anion}(c)}$	$\Lambda^\circ_{\text{anion}(t)}$	$\Lambda^\circ_{\text{anion}(c)} - \Lambda^\circ_{\text{anion}(t)}$
Sodium caseinate.....	100.00	44.20	45.50	-1.30
Calcium ".....	85.20	19.10	†	
Sodium dephosphorized caseinate..	93.00	37.20	37.00	0.20
Calcium ".....	92.50	26.40	11.00	15.40
Monosodium aspartate.....	78.27	27.75	27.60	0.15
Barium diaspertate.....	91.96	27.75	27.50	0.25
Calcium ".....	86.57	27.75	27.70	0.05
Monosodium glutamate.....	75.62	25.10	25.00	0.10
Barium diglutamate.....	89.31	25.10	25.00	0.10
Calcium ".....	83.92	25.10	25.20	-0.10
Aspartic acid.....	377.74	27.75		
Glutamic ".....	375.09	25.10		

\* The values for the casein and the dephosphorized casein compounds are at 30°. The values for the amino acid compounds are at 25°.  $\Lambda^\circ_{\text{anion}(c)}$  is calculated from conductivity measurements;  $\Lambda^\circ_{\text{anion}(t)}$  is calculated from transference experiments. The following values were used for the cation mobilities.

	At 25°	At 30°
$\Lambda^\circ_{\text{Na}^+}$ .....	50.52	55.80
$\Lambda^\circ_{\text{Ca}^{++}}$ .....	58.82	66.10
$\Lambda^\circ_{\text{Ba}^{++}}$ .....	64.21	
$\Lambda^\circ_{\text{H}^+}$ .....	349.99	374.80

† Since, due to the presence of complex ions in the solution, the transference number of calcium caseinate is greater than unity, the calculated value for  $\Lambda^\circ_{\text{anion}(t)}$  is negative. This is an irrational value.

dilution. The data for the mobility of the cation were obtained from the International Critical Tables (20). For sodium, the value 55.80 mhos, and for calcium, 66.10 mhos, was used for the mobilities at 30°. From the transference data of the sodium salts of casein and of dephosphorized casein, the mobility of casein at

following concentrations have been used: 0.1 M for monosodium aspartate and glutamate, 0.05 M for barium diaspertate and diglutamate, 0.04 M for calcium diaspertate and diglutamate.

this temperature was calculated to be 45.5 mhos, and the mobility of dephosphorized casein was 37.0 mhos. These values check quite well with those which were determined from conductivity measurements, *viz.* 44.2 and 37.2, respectively (see Table III).

Examination of the data for the sodium caseinate and sodium dephosphorized caseinate transference experiments shows that the current is carried by the metallic cation and protein anion. In the case of the calcium salts of these two proteins, the transference data are abnormal, indicating that part of the calcium is bound by the protein in a non-ionic form which, as a complex, carries a certain amount of the calcium in a direction opposite to the path of migration of the cation. The formation of such a complex can be expressed by Equation 1 given above. This equation has been used by Greenberg and Schmidt (9) to develop a formula for the transference number of the cation in terms of the fraction of the cation in the form of free ions and the mobilities of the ions in solution as follows:

$$T_{\text{cation}} = \frac{i u - (1 - i) v}{i(u + v)} \quad (3)$$

where  $i$  stands for the fraction of calcium which exists as cation,  $u$  = the mobility of the cations, and  $v$  = the mobility of the complex ions. The value of  $u$ , the cation mobility, is obtained from the International Critical Tables (20). No numerical data are available for  $v$ , the mobility of the complex ion or ions. This value was calculated with the aid of transference and conductivity data. Knowing the total conductivities of the calcium caseinate and of the calcium dephosphorized caseinate solutions, and the mobilities of the calcium ion and of the casein and of the dephosphorized casein ions respectively, and the per cent ionization of the calcium caseinate and of the calcium dephosphorized caseinate, the mobilities of the two respective complex ions were calculated as the difference between the value of  $\Lambda$  for each product and the sum of the mobility of the calcium and the casein or dephosphorized casein ion. The value for the mobility of the calcium-casein complex ion is 32.2 and for the calcium-dephosphorized casein complex ion, 33.0.

Recently, Greene and Power (21) have reported that approximately 11 per cent of sodium, 24 per cent of potassium, and 35 and

45 per cent respectively of calcium and magnesium are bound to certain proteins in a non-diffusible form, thus indicating formation of complexes. Our results with sodium caseinate, as well as those of Greenberg and Schmidt with other alkali metals, do not, within the limits of experimental error which may be assumed to be about 10 per cent, indicate that the alkali metals yield complex ions with casein. Our results indicate that calcium forms complex ions with casein to the extent of about 50 per cent and with dephosphorized casein to the extent of about 40 per cent. The assumption was originally made that the difference between casein and dephosphorized casein was in their content of phosphorus. Our results show that there are about 10 per cent less complex ions in

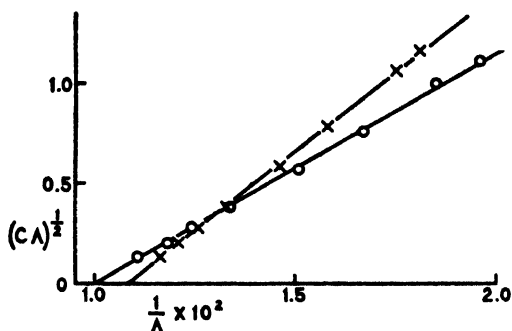


FIG. 1. Conductivity of sodium caseinate, O, and of sodium dephosphorized caseinate, X.

solutions of dephosphorized casein than in casein, or, in other words, that the phosphoric acid in solutions of casein is the seat of complex calcium ions to the extent of about 10 per cent.

The transport data for solutions of the sodium, barium, and calcium salts respectively of glutamic acid and of aspartic acid are given in Table II. Within the range of concentrations studied, and the limits of the experimental method, the data show no evidence of complex ion formation. This is in agreement with the results which were obtained by Kirk and Schmidt (8). It is therefore reasonable to assume that in the native protein molecule the free carboxyl groups do not form complex ions with calcium. However, this point will be discussed later.

The conductivity data for the various salts of casein, dephos-

phorized casein, aspartic and glutamic acids are graphically represented in Figs. 1 to 5. The method of plotting the conductivity

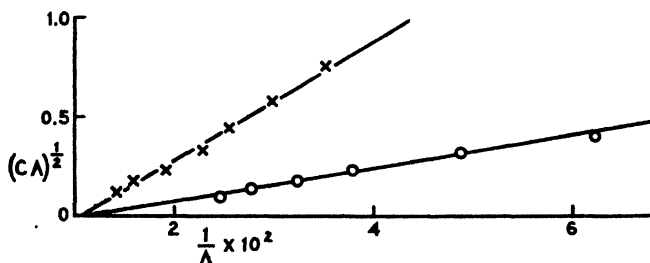


FIG. 2. Conductivity of calcium caseinate, O, and of calcium dephosphorized caseinate, X.

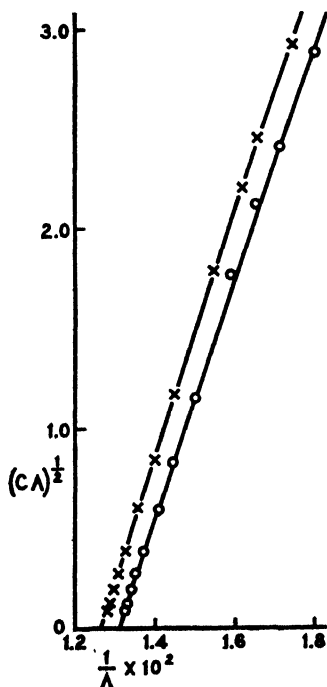


FIG. 3. Conductivity of sodium aspartate, X, and of sodium glutamate, O

data in these figures is the same as that which was used by Greenberg and Schmidt.

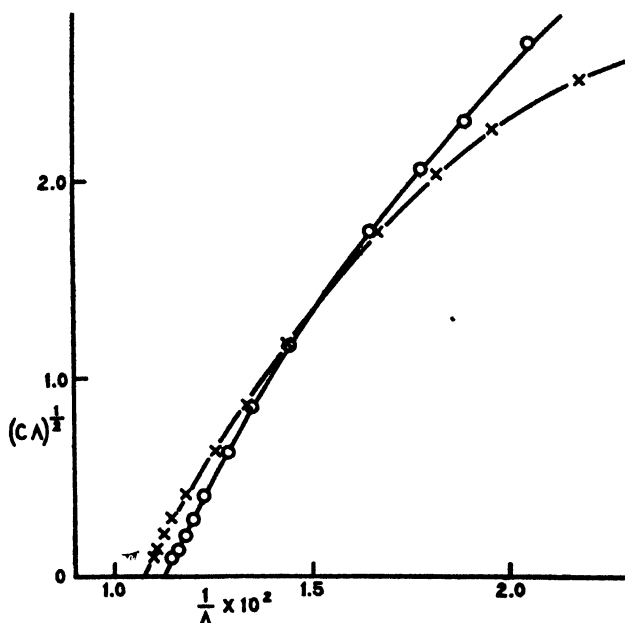


Fig. 4. Conductivity of barium aspartate,  $\times$ , and of barium glutamate,  $\circ$

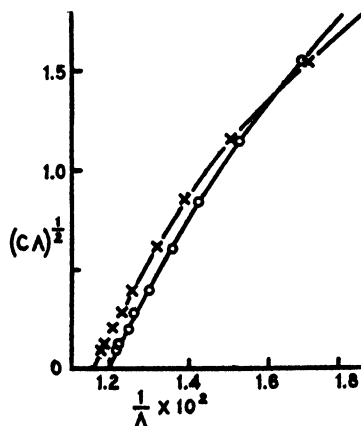


Fig. 5. Conductivity of calcium aspartate,  $\times$ , and of calcium glutamate,  $\circ$ .

In Table III, we have assembled the data for the mobilities of the various anions which have been determined from transference and

from conductivity measurements. With the exception of calcium caseinate and calcium dephosphorized caseinate, the agreement is good. In these two instances, the lack of agreement is due to the presence in the solution of complex calcium-protein ions. Greenberg and Schmidt (5) have already commented on the high value of the mobility of the casein ion. Our results show the same thing. It is of interest to note that the value of the mobility of the casein ion is somewhat larger than the mobility of the dephosphorized casein ion. The values for the mobilities of the amino acid ions are considerably less than similar values for the mobilities of the casein ions or even of the acetate ion (45 mhos).

With the aid of Onsager's (22) modification of the Debye-Hückel (23) theory for the conductivity of strong electrolytes, it has been found possible to plot the conductivity data in another way. The limiting formula for equivalent conductance may be written

$$\Lambda = \Lambda^0 - \alpha \sqrt{\Gamma}$$

where  $\Lambda$  = the molar conductivity and  $\Gamma$  = twice the ionic strength =  $(Z_1 + Z_2 \dots)\mu$ . The term  $\mu$  = molecular concentration, and  $Z_1$  and  $Z_2$  = valences of anions and cations. The term  $\alpha$  is a constant. At 25°, when the solvent is water, theoretical values for  $\alpha$  are given by the equations

$$\alpha = 35.7 + 0.159 \Lambda^0 \text{ (for uni-univalent electrolytes)}$$

$$\alpha = 53.55 + 1.084 \frac{q}{1 + \sqrt{q}} \Lambda^0 \text{ (for uni-bivalent electrolytes)}$$

where  $q = \Lambda^0/(\Lambda^0 + l_1)$  and  $l_1$  is the mobility of the univalent ion. When  $\sqrt{\Gamma}$  is plotted against  $\Lambda$ , the curve will be a straight line. However, Onsager found for a number of strong electrolytes a deviation from a straight line in that the curvature of the curve was upward.

In Figs. 6 to 10, our conductivity data for the sodium, calcium, and barium salts of aspartic acid and of glutamic acid, and the sodium and calcium salts of casein and of dephosphorized casein have been similarly plotted. Our curves have the same general shape as those found for strong electrolytes by Onsager. This may be taken as an indication that the behavior of the amino acids and

the proteins studied by us does not differ materially from the behavior of the strong electrolytes.

In Table IV are given the values for  $\alpha$  for the compounds which we have studied. For the purpose of simplifying the calculations,

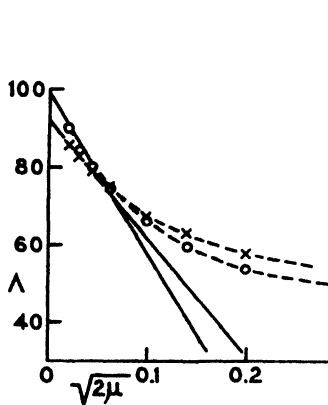


FIG. 6

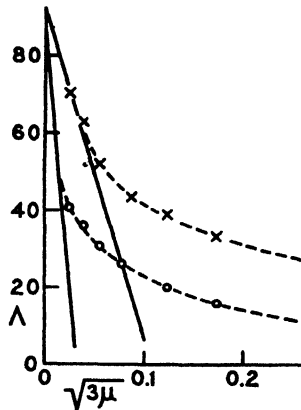


FIG. 7

FIG. 6. Conductivity of sodium caseinate, O, and of sodium dephosphorized caseinate, X, plotted according to Onsager's equation.

FIG. 7. Conductivity of calcium caseinate, O, and of calcium dephosphorized caseinate, X, plotted according to Onsager's equation.

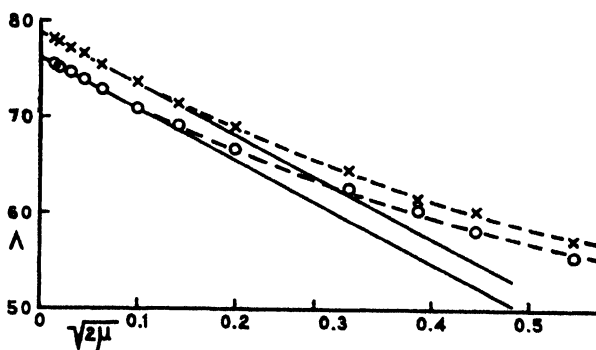


FIG. 8. Conductivity of sodium aspartate, X, and of sodium glutamate, O, plotted according to Onsager's equation.

the assumption was made in the case of casein and of dephosphorized casein that these proteins behave as univalent ions. The same assumption has been made by Kirk and Schmidt (8).

It will be noted that in the case of the salts of aspartic acid and of glutamic acid, the agreement between the experimental and the theoretical values of  $\alpha$  is good. In the case of casein and of

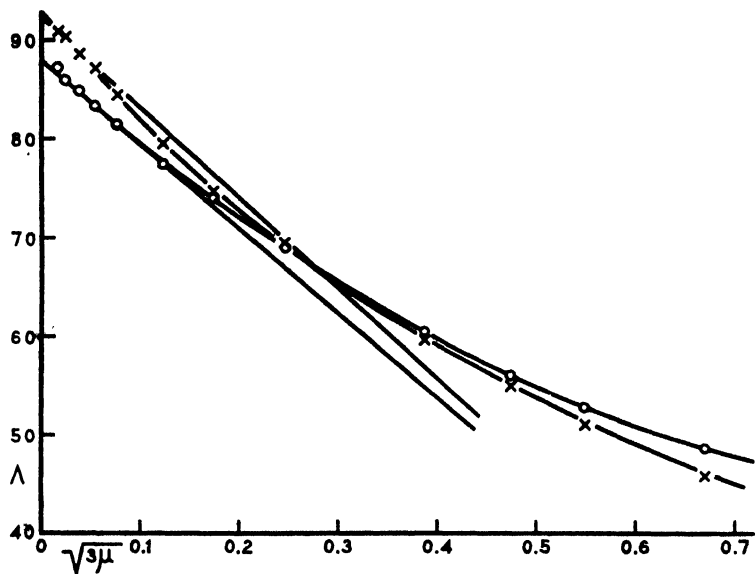


FIG. 9. Conductivity of barium aspartate,  $\times$ , and of barium glutamate,  $\circ$ , plotted according to Onsager's equation.

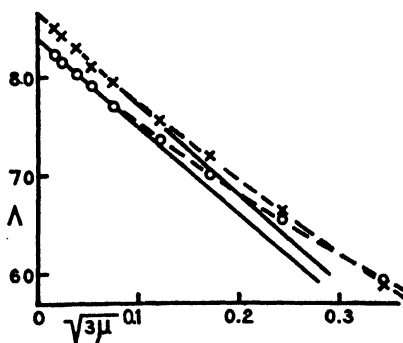


FIG. 10. Conductivity of calcium aspartate,  $\times$ , and of calcium glutamate,  $\circ$ , plotted according to Onsager's equation.

dephosphorized casein, the difference is considerable. While we have no definite explanation to offer for this fact, it is possible that

it may be due to the extensive aggregation of the casein molecules in solution.<sup>1</sup> The difference is most marked in the case of the calcium salts of casein and of dephosphorized casein. This may perhaps be due in part to the presence of the calcium-protein complex ions. Hoskins, Randall, and Schmidt (24) have concluded from freezing point data that ionic micelles are present in solutions of the sodium salts of aspartic and of glutamic acids. The method of plotting the conductivity data of the salts of these

TABLE IV  
*Experimental and Calculated Values for  $\alpha$  in Onsager's Equation*

Salts	$\Lambda^0$	$\alpha_{\text{experimental}}$	$\alpha_{\text{theoretical}}$	$\alpha_{\text{experimental}}$ minus $\alpha_{\text{theoretical}}$
Sodium chloride.....	108.89	54.69	53.00	+1.70
Monosodium glutamate.....	75.62	50.80	47.72	+3.08
"    aspartate.....	78.27	51.70	48.15	+3.55
Barium nitrate.....	116.95	92.80	86.90	+5.90
"    diaspartate.....	91.96	93.30	94.45	-1.15
"    diglutamate.....	89.31	95.30	93.45	+1.65
Calcium chloride.....	116.69	88.00	86.10	+1.90
"    diaspartate.....	86.57	90.80	91.25	-0.45
"    diglutamate.....	83.92	89.80	90.75	-0.95
Sodium caseinate.....	100.00	425.0	51.6	+374.4
"    dephosphorized case- inate.....	93.00	290.0	50.5	+239.5
Calcium caseinate.....	85.20	2750.0	87.0	+2663.0
"    dephosphorized case- inate.....	92.50	870.0	92.3	+777.7

amino acids is apparently not sufficiently sensitive to show this phenomenon.

From the above it will be seen that both casein and dephos-

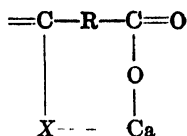
<sup>1</sup> This is offered as a tentative explanation. In doing so and in accepting the suggestion of Hoskins, Randall, and Schmidt (24), the writers are fully aware of the limitations contained therein. It is obvious that the suggestion does not account for the forces involved nor the mechanism whereby micelles are formed. The presence of micelles in a solution is not, however, to be regarded as evidence for a static system, but rather a dynamic one. It is obvious that freezing point data and data of a like nature are merely statistical averages.

phorized casein form complex ions with calcium, the latter compound about 10 per cent less than the former. Evidently, some of the calcium is bound to the phosphoric acid of casein and is carried in a non-dissociated form. Since glutamic and aspartic acids do not yield complex ions with calcium, we cannot attribute the seat of complex ion formation to the free carboxyl groups. It is not probable that the hydroxy group of  $\beta$ -hydroxyglutamic acid is concerned with the complex ion formation since Shear and Kramer (25) have shown that lactic acid does not yield complex ions with calcium. On the other hand, calcium appears to yield complex ions with citric acid. However, we know of no groups in the casein molecule similar to those which are present in citric acid.

Since within the range of acidity at which our experiments have been carried out the calcium is largely, if not entirely, bound to the free carboxyl groups, we are forced to look to these groups for the seat of complex ion formation. On account of the large number of free carboxyl groups, casein can be considered as a polybasic acid. It is conceivable that the calcium which is bound to certain of the free carboxyl groups is more easily ionized than that which is bound to the remainder. The net result is step dissociation of the type which takes place in polybasic acids.<sup>2</sup> If this be true, the non-ionized calcium which is bound to carboxyl groups will, in transference experiments, be carried in a direction opposite to the path of migration of the dissociated calcium ions. *A priori*, according to this hypothesis, there is no reason why the alkali metals should not likewise yield complex ions with proteins. It is quite possible that a small number of such ions are formed. The number must, however, be within the limits of error of our transference experiments. The net difference between the ionization of casein which is combined with the alkali metals and that which is combined with the alkaline earths is one of degree rather than one of kind. It is not possible at the present time to exclude the effect which secondary valence may play in the formation of complex ions between protein and the

<sup>2</sup> In this connection see Pauli, W., and Valkó, E., *Elektrochemie der Kolloide*, Vienna, 417 (1929).

alkaline earth elements. It is possible that compounds of the type



in which  $X$  is an amino group, hydroxyl group, or other unknown group may be formed. Our experiments, however, do not give any indications of the formation of such complexes.

We have incidentally determined the conductivity of solutions of free aspartic acid, glutamic acid, and glycine. The data are given in Table V and for the first two mentioned amino acids are shown graphically in Figs. 11 and 12. These measurements throw some light on the electrochemical behavior of solutions of these amino acids.

In 1904, Walker (26) showed that the relations between the acidity of a solution of an amphoteric electrolyte and the dissociation constants could be expressed by the equation

$$(\text{H}^+)^2 = \frac{K_w + K_a u}{1 + K_b u / K_w} \quad (4)$$

in which  $K_a$  and  $K_b$  are the classical acid and basic dissociation constants respectively of the amphoteric substance,  $u$  is the concentration of the undissociated molecule, and  $K_w$  is the dissociation constant of water. Walker stated "when  $K_b/K_w$  and  $u$  have finite values it is obvious that amphoteric electrolytes cannot strictly obey Ostwald's dilution law. If, however, either  $K_b/K_w$  or  $u$  is very small, Ostwald's dilution law is approximately followed, for then the values of  $\text{H}^+$  from the simple and amphoteric formulæ become nearly equal, . . . The smaller the basic dissociation constant, then, and the greater the dilution, the more likely is the amphoteric electrolyte to follow the dilution law characteristic of simple acids and bases."<sup>3</sup>

Now Ostwald's dilution law is derived from the following considerations (27). On the assumption of constant ionic mobility

<sup>3</sup>For the sake of uniformity the symbols in this quotation have been changed to conform to our usage.

$\Lambda/\Lambda^0$  gives the degree of dissociation at each concentration and  $m\Lambda/\Lambda^0$  gives the molality of each ion, while  $m(1 - \Lambda/\Lambda^0)$  gives the molality of the undissociated substance. Now

$$K_A = \frac{m(\Lambda/\Lambda^0)^2}{(1 - \Lambda/\Lambda^0)} \quad (5)$$

If in a reaction of the type  $XY = X^+ + Y^-$  we assume that the activity of each substance is equal to its molality, then  $K_A = K$ . This expresses Ostwald's dilution law.

Walker (26), using Winkelblech's conductivity data for amino-benzoic acid, showed that on account of the factors which have been quoted above, Ostwald's dilution law was only approximated. Acetic acid is known to obey Ostwald's dilution law even in fairly high (0.13 M) concentration. On the other hand, Johnston (28) found that cacodylic acid, the dissociation constant of which is considerably smaller than that of acetic acid, obeys Ostwald's dilution law only approximately. Robertson (29) found that solutions of certain proteins in various alkalies and acids followed the dilution law quite well. In his calculations he was forced to make the assumption that the equivalent concentration of the base or acid which is neutralized by the protein, bears a constant proportion to the true equivalent concentration of the protein salt. No attempts to apply the Ostwald dilution law to aminocarboxylic acids have apparently been made.

In Table V are given the calculated values for  $K_A$  and the experimental values for  $K$ , the dissociation constants of which have been determined electrometrically. We have made the assumption that the apparent dissociation constant can be used in place of the true dissociation constant. In the case of glutamic and aspartic acids this assumption has been shown by Miyamoto and Schmidt (30) to be justifiable. It will be seen that although the values of the acid dissociation constant of aspartic acid and of glutamic acid are essentially the same as that of acetic acid, the value for  $K_A$  of these two amino acids does not approach a constant value even at a 0.0001 M dilution, whereas the value of  $K_A$  for acetic acid is a constant at 0.13 M. The tendency for the values for  $K_A$  of all three amino acids is to become constant only at extremely high dilutions. It is of interest to note that whereas the values for  $K_A$  of aspartic and of glutamic acids are smaller in high concentrations

TABLE V

*Conductivity Data of Aspartic Acid, Glutamic Acid, and Glycine at 25°*

	<i>m</i>	<i>L</i> × 10 <sup>4</sup>	$\Delta$	$\alpha^*$	$K_A \times 10^4$
Aspartic acid	0.0000		377.74	1.000	1.50
	0.0001	2.450	245.00	0.649	1.19
	0.0002	3.940	197.00	0.522	1.14
	0.0005	7.048	140.96	0.373	1.12
	0.001	10.67	106.70	0.283	1.12
	0.002	15.61	78.05	0.207	1.08
	0.005	24.33	48.66	0.129	0.955
	0.01	32.69	32.69	0.0867	0.823
	0.02	43.41	21.70	0.0576	0.704
	0.04	58.38	14.60	0.0387	0.623
					$K_A \times 10^5$
Glutamic acid	0.0000		375.09	1.000	5.62
	0.0001	1.782	178.20	0.475	4.31
	0.0002	2.760	138.00	0.368	4.28
	0.0005	4.651	93.12	0.248	4.13
	0.001	6.641	66.41	0.181	3.99
	0.002	9.461	47.31	0.126	3.63
	0.005	14.30	28.60	0.0763	3.39
	0.01	18.90	18.90	0.0504	2.68
	0.02	25.15	12.58	0.0335	2.35
	0.04	34.74	8.69	0.0232	2.22
	0.05	39.09	7.82	0.0209	2.21
		<i>L</i> × 10 <sup>4</sup>		$\alpha \times 10^4$	$K_A \times 10^{10}$
Glycine	0.00		380.0000†		2.54
	0.01	1.183	0.1183	3.12	9.75
	0.02	2.124	0.1062	2.80	15.70
	0.05	3.818	0.07636	2.00	20.00
	0.1	6.978	0.06978	1.87	35.00
	0.2	13.62	0.06810	1.79	64.00
	0.5	26.81	0.05362	1.41	99.50
	1.0	50.88	0.05088	1.34	179.50

The dissociation constant,  $K$ , of the amino acids given in Table V has been taken from the table of Miyamoto and Schmidt (30). However, the dissociation constant of aspartic acid given in their table is the one calculated from the data of ( $\Delta H$ ) at 25°. We, therefore, have chosen the value of Lundén (31) for aspartic acid.

\*  $\alpha$  (classical degree of dissociation) =  $\Delta/\Delta^0$ .

† The mobility of the glycine ion is assumed to have the value 30.

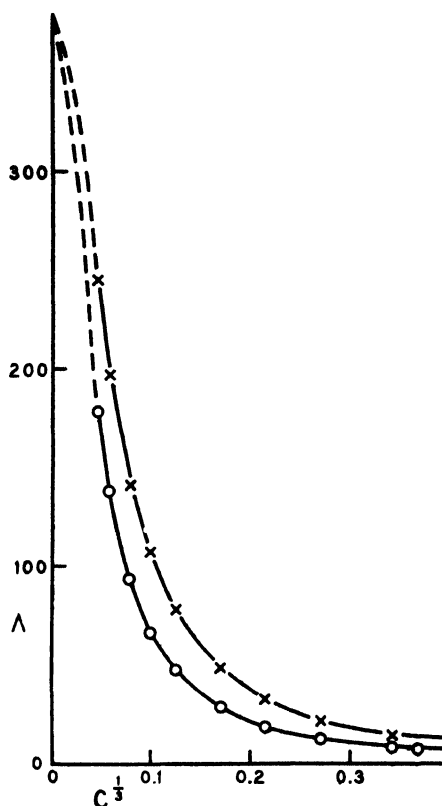


FIG. 11. Conductivity of aspartic acid, X, and of glutamic acid, O

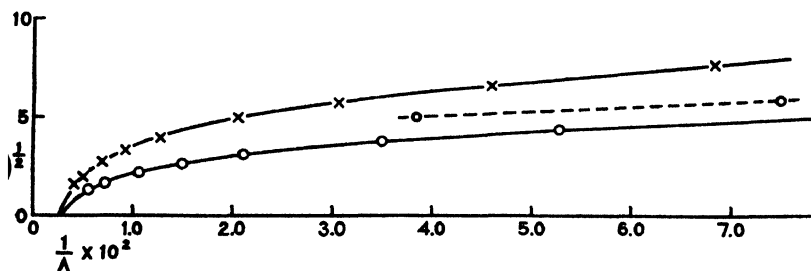


FIG. 12. Conductivity of aspartic acid, X, and of glutamic acid, O. The dotted line is the extension of the lower curve. The values of the ordinates are as given. The values of the abscissæ must be increased by adding 4 to the values given, for the extension of the curve.

and tend to become larger as the dilution increases, the opposite is true for glycine.

The data show that these three amino acids do not strictly obey Ostwald's dilution law, due apparently to the factors which have been pointed out by Walker.

The numerical data which were used to plot the curves contained in this paper are on file in the University of California library.

#### SUMMARY

1. Transference experiments with solutions of certain salts of casein, dephosphorized casein, glutamic acid, and aspartic acid have been carried out.

2. Conductivity measurements have been carried out on solutions of certain salts of casein, dephosphorized casein, aspartic acid, and glutamic acid. The conductivities of aspartic acid, glutamic acid, and glycine have also been determined.

3. The data show that within the limits of accuracy of the transference experiments, the sodium salts of casein, dephosphorized casein, aspartic acid, and glutamic acid, and the calcium and barium salts of the two last mentioned amino acids do not yield complex ions; whereas, solutions of the calcium salts of casein and of dephosphorized casein yield complex ions in solution.

4. It has been shown that the number of complex ions in solutions of calcium dephosphorized casein is about 10 per cent less than in solutions of calcium caseinate. It is concluded that the phosphoric acid in casein is responsible to the extent of about 10 per cent for the complex ions in solutions of calcium caseinate.

5. The hypothesis has been advanced that solutions of casein in the alkaline earth elements yield complex ions due to step dissociation.

6. The method of plotting conductivity data as proposed by Onsager has been applied to the conductivity data of the salts of aspartic acid, glutamic acid, casein, and dephosphorized casein. Comparisons have been made between these curves and the curves for similarly plotted strong electrolytes.

7. Attempts have been made to apply Ostwald's dilution law to solutions of aspartic acid, glutamic acid, and glycine. The results indicate that these amino acids tend to obey the dilution law only in extremely high dilutions.

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## THE EFFECT OF TEMPERATURE ON THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD

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In the course of studies of the carbon dioxide absorption curve of human blood which were published from this laboratory in 1923 and 1924 (1-3) a few data on the effect of temperature were collected. Since these results were suggestive and since temperature ranks as one of the important determinants of the curve, it was decided to extend the studies to include the rôle of temperature.

An inevitable effect of increased temperature is a reduction of the physically dissolved  $\text{CO}_2$ . It is of theoretical and practical importance to find out how this decrease in  $\text{H}_2\text{CO}_3$  affects total  $\text{CO}_2$ , bicarbonate, and pH of the blood. Temperature also determines the dissociation of the ampholytes of the blood, chief among which are hemoglobin and serum proteins. Changes in the dissociation constants of these substances involve alterations of their base-binding capacities, and since bicarbonate is the most important available competitor for base, it is to be expected that the amount of base which will combine with bicarbonate is a function of the temperature, other factors remaining constant.

From the physiological standpoint, investigation of temperature effects should throw some light on the acid-base equilibrium in fever. Finally, it would be of practical convenience to be able to predict from the  $\text{CO}_2$  content of blood saturated at other temperatures its  $\text{CO}_2$  content at body temperature.

The most important previous studies of the effects of temperature on acid-base equilibrium were concerned with pH and  $\text{pK}_1$ ,

\* Some of the data in this paper are taken from a dissertation submitted by Anna J. Eisenman in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1928.

the slope and height of the absorption curve, and the relation of serum curves to blood curves.

Investigations of the effect of temperature on pH have yielded divergent results (4-12). There have been three well substantiated determinations of the effect of temperature on  $pK_1$  (Stadie and Martin (7), Warburg (12), and Cullen, Keeler, and Robinson (13)). The effect of temperature on the slope and height of the absorption curves and on the relation of serum curves was investigated by Stadie *et al.* (8), Austin and Cullen (9), Christiansen *et al.* (14), Van Slyke and Cullen (15), and Haggard (16).

#### EXPERIMENTAL

##### *Methods*

All blood was collected without stasis because stasis had been found by Morawitz and Walker (17) to alter the CO<sub>2</sub> content and CO<sub>2</sub>-combining power of blood, and by Peters, Eisenman, and Bulger (18) to affect cell and serum volume. In all but two experiments, the blood was collected without precautions against exposure to air.

In Experiments 4 to 9 coagulation of blood was prevented by the addition of 200 mg. of neutral potassium oxalate per 100 cc. of blood. After it had been definitely established (19) that potassium oxalate disturbed the electrolyte equilibrium of blood, the blood was defibrinated by stirring with a glass rod. The receiver containing the blood was placed in a beaker of ice water until ready for equilibration (1). In all of the experiments except the most complicated ones, the blood was equilibrated within from 15 to 60 minutes after its withdrawal.

Completely oxygenated blood was used throughout the series of experiments. Saturation with CO<sub>2</sub> was carried out according to the method described by Austin *et al.* (20) as "Second saturation method," with 750 cc. tonometers (1). In a very few experiments, saturations at the lower temperature were carried out by rotating the tonometers in the air in a sheltered part of the room. In all other cases, however, the tonometers were rotated in a constant temperature water bath in which the desired temperature was maintained within 0.2°. All equilibrations were at 40 mm. of CO<sub>2</sub> in air unless otherwise specified.

After saturation the blood was transferred to sampling bulbs

over mercury and was centrifuged in completely filled stoppered tubes, from which the serum was removed to similar bulbs with all the precautions to avoid loss of  $\text{CO}_2$  described by Austin *et al.* (20).

*Cell Volume*—This was measured by means of the ordinary Daland hematocrit fitted to a No. 1 International Equipment Company centrifuge. This method seems to have an error not exceeding 1 per cent (21, 22).

*Oxygen Capacity*—In all except a few of the earlier experiments, this was determined by the carbon monoxide method of Van Slyke and Hiller (23).

*Carbon Dioxide*—This was determined either by the Van Slyke and Stadie (24) method in the constant pressure Van Slyke burette or by the Van Slyke and Neill (25) method in the constant volume apparatus.

*Carbonic Acid*—Carbonic acid was calculated by means of the solubility coefficient obtained by Bohr (26). He estimated that the solubility of  $\text{CO}_2$  in serum was 97.5 per cent and in cells 81 per cent, of its solubility in water. The water solubility coefficients of Bohr and Bock (27) were used. Bohr's coefficients have been generally superseded by those of Van Slyke, Sendroy, Hastings, and Neill (28). The latter could not be employed in these studies, because they were determined only at  $38^\circ$ . Regardless of which solubility coefficients are used, the comparative results will be the same, since carbon dioxide and bicarbonate are related as linear functions at constant  $\text{CO}_2$  tension.

### Results

*Relation of Effect of Temperature to Oxygen Capacity*—The first experiments were carried out at varying intervals of temperature. The divergent results obtained suggested the wisdom of limiting the number of variables. Accordingly, a series of experiments upon the effects of a definite temperature interval of  $38\text{--}23^\circ$  was undertaken. The data from these thirty-six experiments are presented in Table I. In Fig. 1,  $\Delta[\text{BHCO}_3]_{23\text{--}38^\circ}$  is plotted against oxygen capacity, which varies from 3.4 to 25.3 volumes per cent.

There is a general tendency for  $\Delta[\text{BHCO}_3]_{23\text{--}38^\circ}$  to increase with increasing oxygen capacity. It will be noted that two points, those from Experiments 37-2 and 24-2, lie somewhat above, and one point, from Experiment 21-2, lies below, the general number of



Primary anemia.....	41	16.0	37.6	40.5	43.5	56.6	13.1	11.8	+1.3	7.35	7.38
Secondary ".....	26	16.6	38.0	38.7	53.2	66.4	13.2	11.8	+1.4	7.42	7.45
Normal.....	17-N	18.0	40.8	37.7	40.5	53.3	12.8	12.5	+0.3	7.33	7.35
" *.....	14	18.7			42.2	53.9	11.7	12.7	-1.0	7.34	7.36
".....	23	19.0	43.4	43.5	43.4	54.9	11.5	12.8	-1.3	7.36	7.37
Primary anemia after treatment.....	37-2	19.5	52.0	54.5	44.2	59.9	15.7	12.9	+2.8	7.37	7.42
Polycythemia ".....	21-2	19.6	45.4	46.5	46.7	57.3	10.6	13.0	-2.4	7.39	7.39
Normal*.....	15	20.7			46.1	59.0	12.9	13.3	-0.4	7.39	7.41
".....	22	21.1	51.1	50.5	39.6	53.9	14.3	13.4	+0.9	7.32	7.37
Primary anemia after treatment.....	39	21.1	48.3	48.7	45.2	59.1	13.9	13.4	+0.5	7.34	7.41
Normal.....	9	21.3	48.7		43.8	57.6	13.8	13.5	+0.3	7.37	7.40
".....	16-N	21.3	50.8	50.1	42.3	55.6	13.4	13.5	-0.1	7.35	7.38
".....	20	23.2	50.3	50.5	36.4	50.6	14.2	14.1	+0.1	7.29	7.34
Hypothyroid.....	31	22.6	52.1	57.0	44.7	59.2	14.5	13.9	+0.6	7.39	7.42
Normal.....	10	23.7	55.6	54.9	36.6	50.5	13.9	14.3	-0.4	7.29	7.34
Polycythemia.....	21-1	24.2	55.0	55.3	40.3	54.4	14.1	14.4	-0.3	7.33	7.37
".....	11	24.3	56.2	54.8	38.6	52.6	14.0	14.5	-0.5	7.31	7.37
".....	12	24.5	58.6	59.1	38.0	51.9	13.9	14.5	-0.6	7.31	7.34
".....	40	24.7	70.3	69.5	38.6	54.7	16.2	14.6	+1.2	7.33	7.40
".....	42	25.3	60.3	61.5	39.7	53.9	14.2	14.8	-0.6	7.33	7.38

\* The data for Cases 14 and 15 were secured by calculating  $[\text{BHCO}_2]$  at 40 mm. of  $\text{CO}_2$  tension from analyses at 30 and 60 mm.

the points. The first are from cases of primary anemia, treated for a period of months; the last one from a treated case of polycythemia. No explanation is offered for the failure of blood from treated cases of polycythemia and anemia to behave like that from untreated cases.

It is not apparent from the grouping of the points whether the relation between oxygen capacity and  $\Delta$  is a rectilinear one or whether the curve flattens out in the region of high hemoglobin to become asymptotic to the  $x$  axis. In order to throw light on this

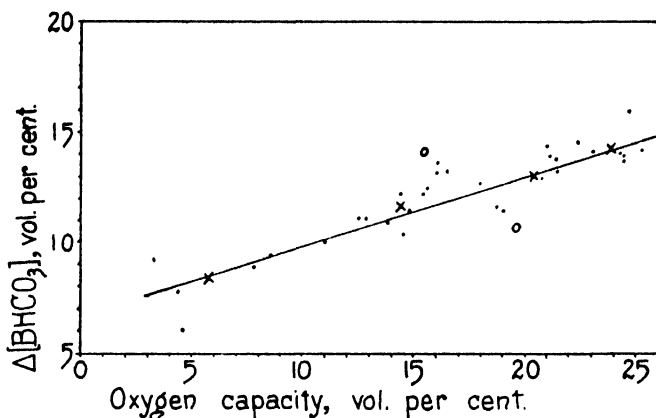


FIG. 1. The relation of the change of blood bicarbonate between 23–38° to the oxygen capacity of blood. The circles represent studies on treated cases of pernicious anemia and polycythemia; the crosses, group averages. The equation of the line describing the distribution of the points is  $\Delta[\text{BHCO}_3]_{23-38^\circ} = (0.32 \text{ oxygen capacity} + 6.7) \text{ volumes per cent.}$

issue, the values were averaged in groups according to oxygen capacity: 0 to 10, 10 to 18, 18 to 22, and 22+ volumes per cent. The three aberrant points mentioned above were excluded from these averages. The four average points lie along a straight line having the equation  $\Delta[\text{BHCO}_3]_{23-38^\circ} = (0.32 \text{ oxygen capacity} + 6.7) \text{ volumes per cent.}$  This relationship is not significantly altered by the inclusion of some eight additional values obtained from artificial polycythemias and anemias.

Confirmation of the fact that hemoglobin concentration affects the value of  $\Delta[\text{BHCO}_3]_{t_1-t_2}$  can be obtained from analysis of Stadie, Austin, and Robinson's (8) data on whole blood. Their calcu-

lated values of  $\Delta[\text{BHCO}_3]/\Delta t$ ,  $\text{CO}_2$  tension remaining constant, vary in the same direction as oxygen capacity.

It was thought that if the data from a heterogeneous collection of bloods showed so fair an approximation to a rectilinear relationship between hemoglobin and  $\Delta[\text{BHCO}_3]_{23-38^\circ}$ , an exact relationship might be demonstrated in any one blood. It has already been shown (3) that in any one blood hemoglobin content bears a fairly exact relationship to  $\text{CO}_2$  capacity at  $38^\circ$ . If this relationship

TABLE II  
*Rectilinear Relationship of Hemoglobin to Bicarbonate Content*

Experiment No.	Specimen	Oxygen capacity	Bicarbonate content		$\Delta[\text{BHCO}_3]_{23-38^\circ}$
			38°	23°	
		vol. per cent	vol. per cent	vol. per cent	vol. per cent
16. Aerobic	Blood A	14.4	47.5	59.4	11.9
	" N	21.3	42.2	55.6	13.4
	" P	27.3	36.9	50.6	13.7
17. Anaerobic	" A	10.0	45.5	55.5	10.0
	" N	18.0	40.5	53.3	12.8
	" P	25.3	37.4	50.9	13.5
43. "	True serum	0	51.9		
	Blood AA	3.4	50.8		
	" A	17.7	42.7		
	" N	22.1	40.1		
	" P	27.8	37.5		
44. "	" AA	3.6		65.4	
	" A	15.0		58.8	
	" N	23.6		53.9	
	" P	31.6		50.6	

also holds at  $23^\circ$ , then  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  must be related to hemoglobin. Accordingly, two experiments were performed in which normal blood was rendered anemic or polycythemic by adding to the cells, or removing from them, some of their own serum (Experiments 16 and 17, Table II). The bloods were then saturated with 40 mm. of  $\text{CO}_2$  at  $23^\circ$  and at  $38^\circ$ . The bicarbonate-hemoglobin graphs for these two experiments may be seen in Fig. 2. The relationship at  $23^\circ$  seems not less exactly linear than that at  $38^\circ$ . At both temperatures hemoglobin seems to bear a straight line relationship to bicarbonate content. Since the effect of hemoglobin

is greater at one temperature (38°),  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  must increase with hemoglobin.

It was further possible by means of completely anaerobic experiments (Table II and Fig. 3) to prove that the relation between hemoglobin and CO<sub>2</sub> capacity at any one temperature is not only approximately, but is exactly rectilinear.

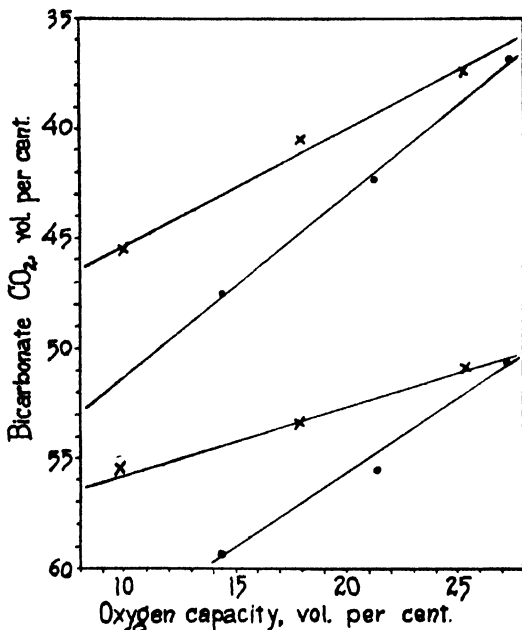


FIG. 2. Variations of bicarbonate with oxygen capacity at 38° and 23° in blood samples in which oxygen capacity was varied by using different proportions of cells and serum. The top curve represents Experiment 17 at 38°; the second, Experiment 16 at 38°; the third, Experiment 17 at 23°; and the bottom curve, Experiment 16 at 23°. Crosses and dots indicate determined points.

*Experiment 43*—120 cc. of blood were anaerobically collected and placed over mercury in sampling bulbs containing 240 mg. of potassium oxalate and 120 mg. of sodium fluoride. Two 60 cc. samples of blood were then saturated once with 40 mm. of CO<sub>2</sub> at 38°. After saturation the blood was delivered in varying amounts into four sampling bulbs over mercury and 50 cc. were delivered

under oil into a centrifuge tube. The latter tube was centrifuged. 18 cc. of serum were drawn into sampling Bulb 1, containing 2 cc. of blood (Sample AA). 4 cc. of serum were drawn into sampling Bulb 2, containing 8 cc. of blood (Sample A). Sampling Bulb 3 contained 20 cc. of blood (Sample N). 20 cc. of cells were drawn

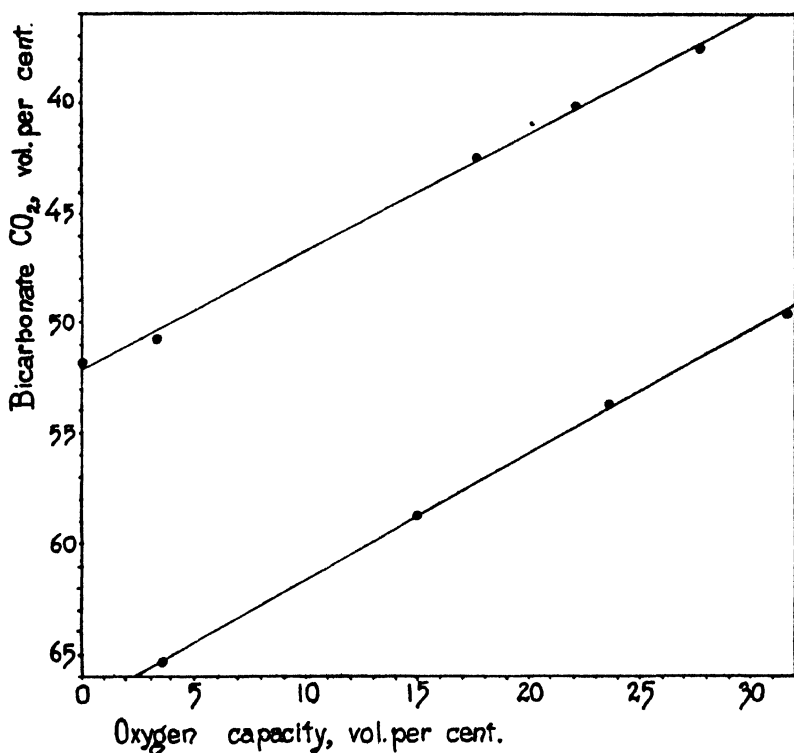


FIG. 3. Variations of blood bicarbonate with oxygen capacity when the latter was altered by using different proportions of cells and serum. The upper curve represents Experiment 43; the lower, Experiment 44. Dots indicate determined points.

into sampling Bulb 4 containing 30 cc. of blood (Sample P). All samples were thoroughly mixed. They afforded four samples of blood, differing from one another only by their relative proportions of serum and cells. From 10 to 18 cc. of these samples were saturated separately once at 40 mm. and 38°, and then their oxygen

capacities, cell volumes, and CO<sub>2</sub> contents determined. Some of the blood from Sample N was then centrifuged, and the true serum analyzed for CO<sub>2</sub> content. The best straight line that can be drawn through these five points has the equation  $\text{BHCO}_3 = (-0.533 \text{ O}_2 \text{ capacity} + 52.1) \text{ volumes per cent}$ . No one point lies more than 0.4 volume per cent from the observed value.

*Experiment 44*—Blood was treated exactly as in Experiment 43, except that the equilibrations were at 23° instead of 38°. The true serum CO<sub>2</sub> content, as determined, was less than that of the anemic specimen. Possibly there was unconscious exposure to air during centrifugation. However, there is a four point curve relating hemoglobin content to CO<sub>2</sub> content. The equation of this curve is  $[\text{BHCO}_3] = (-0.533 \text{ O}_2 + 67.1) \text{ volumes per cent}$ . Again each point is within 0.4 volume per cent of the analytical value.

There were additional two or three point experiments of this character which also confirm the observation that the CO<sub>2</sub> capacity is a linear function of the hemoglobin content. The fact that the slopes of Experiments 43 and 44 are the same is of no significance. Blood from different subjects was used in the two experiments. Experiments 16 and 17 show that if the same blood is saturated at 23° and at 38°, the slope of the 38° curve is greater, while the height of the 23° curve is greater.

The conclusions from these last four experiments are: (1) In any one blood at any one temperature, bicarbonate content is inversely proportional to the hemoglobin content. This relationship is exactly rectilinear. (2) For any one blood, the slope of the 38° curve is definitely greater than that of the 23° curve. (3) It follows that the difference between bicarbonate content at 23° and 38° must always increase with hemoglobin content, and that  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  is a rectilinear function of hemoglobin content.

Since  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  and hemoglobin in any one blood bear such an exact relation, it seemed desirable to see how well this relation would hold for the heterogeneous collection of values in Table I. The values of  $\Delta$  for each experiment were calculated from the equation of the straight line joining the four statistical points. This equation is  $\Delta[\text{BHCO}_3]_{23-38^\circ} = (0.32 \text{ oxygen capacity} + 6.7) \text{ volumes per cent}$ . The calculated  $\Delta$  appears in Column 8 of Table I. The difference between calculated and observed  $\Delta$

appears in Column 9. The two values are close enough to permit rough estimation of  $\text{CO}_2$  capacity at  $38^\circ$  from  $\text{CO}_2$  capacity at  $23^\circ$ . The practical value of this lies in the fact that it permits one to analyze blood saturated at room temperature and from the results to calculate the  $\text{CO}_2$  capacity at  $38^\circ$ .

There are a few experiments (Table III) in which the lower temperature was other than  $23^\circ$ . An attempt was made to correlate the changes from these smaller temperature intervals with those from the  $15^\circ$  interval. In Fig. 4,  $\Delta[\text{BHCO}_3]_{\text{r.t.}-38^\circ}$  is plotted against  $t^\circ$  (r.t. indicates room temperature other than  $23^\circ$ ). There are so few determined points that it is difficult to

TABLE III  
*Effect of Varying Temperature Intervals on Bicarbonate Content*

Experiment No.	Oxygen capacity	High temperature	Low temperature	Whole blood bicarbonate		$\Delta[\text{BHCO}_3]_{\text{r.t.}-38^\circ}$
				High temperature	Low temperature	
	vol. per cent	$^\circ\text{C.}$	$^\circ\text{C.}$	vol. per cent	vol. per cent	vol. per cent
4	10.8	38	25.5	45.8	52.6	6.9
5	15.1	38	22.5	33.6	45.0	11.4
6	18.9	38	25.0	41.8	53.6	11.9
7	18.4	38	26.8	40.1	48.3	8.3
8	19.1	38	30.2	45.0	49.6	4.6
12	24.5	38	30.0	38.0	44.4	6.4

\* The subscript r.t. represents the low temperature in preceding columns.

define any relationship. From the equation of the curve in Fig. 1,  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  can be calculated for various average oxygen capacities. These values are marked off on the  $y$  axis of Fig. 4. Given the oxygen capacity, a straight line passing through the corresponding value for  $(\Delta[\text{BHCO}_3]_{23-38^\circ})$  and  $(x = 38^\circ, y = 0)$  should pass through  $\Delta[\text{BHCO}_3]_{\text{r.t.}-38^\circ}$  if the rate of change in  $\Delta$  per degree change in temperature is regular. It is obvious that such straight lines will not pass through the determined points in Table III. Evidently the increment in  $\Delta$  is not a rectilinear function of the extent of temperature change in this series of bloods.

The question arises whether, in any one blood, there is a rectilinear relationship between  $\Delta[\text{BHCO}_3]$  and  $\Delta t$ . One critical

experiment (No. 12) was performed to settle this point. The same blood was studied at three temperatures, 23°, 30°, and 38°. The rise in bicarbonate per degree of temperature change was

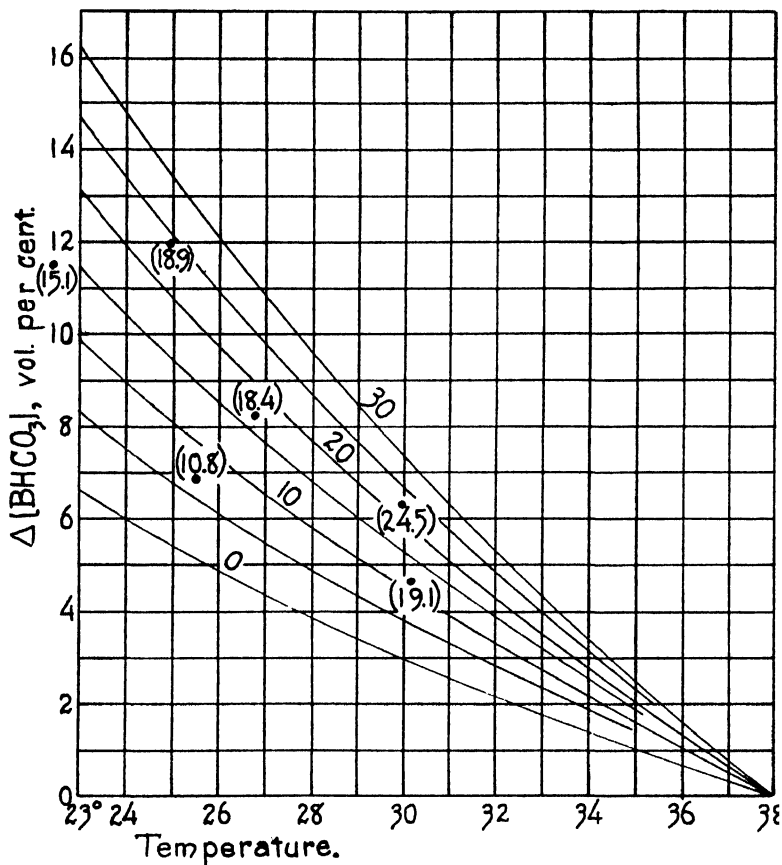


FIG. 4. Chart to estimate blood bicarbonate at 38° from blood bicarbonate determined at temperatures between 23–38°. The curved lines indicate varying oxygen capacity. The individual points represent experiments in which temperature intervals other than 23–38° were employed. The numerals in parentheses indicate the oxygen capacity of these blood samples.

greater between 23–30° than between 30–38°. This suggests that the curve relating  $\Delta[\text{BHCO}_3]$  to  $\Delta t$  for a given oxygen capacity is steeper in the lower temperature intervals than in the intervals about 38°.

In Table III there are three experiments in which the oxygen capacity is about 18 volumes per cent. If these three points are averaged, we obtain the values: oxygen capacity = 18.8 volumes per cent,  $\Delta[\text{BHCO}_3]_{27.3-38^\circ} = 8.27$  volumes per cent. In Fig. 1, at oxygen capacity 18 volumes per cent,  $\Delta[\text{BHCO}_3]_{23-38^\circ} = 12.5$  volumes per cent. A smooth curve drawn through  $x = 23$ ,  $y = 12.5$ ,  $x = 27.3$ ,  $y = 8.27$ , and  $x = 38$ ,  $y = 0$ , takes a form convex to the axis and lies not far from the determined points of these three experiments. This curve should represent the values of  $\Delta[\text{BHCO}_3]$  for any temperature between 23–38°, if the oxygen capacity of the blood studied is 18 volumes per cent. Contour lines for other oxygen capacities are drawn through the appropriate values of  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  and  $x = 38^\circ$ ,  $y = 0$ . These contour lines represent equal increments of hemoglobin. It seems only reasonable to assume that for any given temperature between 23–38°,  $\Delta[\text{BHCO}_3]_{t-38^\circ}$  is a rectilinear function of oxygen capacity. Therefore, these contour lines are an equal distance from each other at each value of  $x$ . The determined values from Experiments 4, 5, and 12 lie near enough to the appropriate oxygen capacity contour lines to make it reasonably certain that the relationship between oxygen capacity,  $\Delta t$ , and  $\Delta[\text{BHCO}_3]$  is expressed by graphs at least similar to those of Fig. 4. There are too few experiments to define the exact shape of the curves. More experiments of this type are contemplated, and their data should result in a method for the estimation of the  $\text{CO}_2$  capacity at 38° from  $\text{CO}_2$  capacity determined at any other temperature reasonably close to 38°.

At present it can only be pointed out that the construction of Fig. 4 satisfies the known facts about the relationships of the three variables: first, that  $\Delta[\text{BHCO}_3]_{t-38^\circ}$  is a linear function of hemoglobin content, that  $\Delta[\text{BHCO}_3]_{23-30^\circ}$  is greater than  $\Delta[\text{BHCO}_3]_{30-38^\circ}$ .

*Effect of Temperature on  $\text{CO}_2$  Capacity of True Serum and Cells*—There are five experiments (Table IV) in which the  $\text{CO}_2$  capacity of true serum was determined at two temperatures, 23° and 38°. In Experiments 14 and 15 the blood was saturated at 30 and 60 mm. and the 40 mm. values for blood and serum were calculated (2). The hematocrit figures used are the means of the 30 and 60 mm. values.

No definite temperature relationships for true serum can be

TABLE IV  
*Effect of Temperature on Bicarbonate of Cells and Serum*

Experiment No.	Oxy- gen capac- ity	Cell volume				Bicarbonate						$\Delta[\text{HCO}_3]_{12-38^\circ}$			1/D		$\frac{\Delta[\text{HCO}_3]_{TS}}{\Delta[\text{HCO}_3]_B}$	$\frac{\Delta[\text{HCO}_3]_{TS}}{\Delta[\text{HCO}_3]_C}$
		38°		23°		Whole blood		True serum		Cells		Whole blood	True serum	Cells				
						38°	23°	vol. per cent	vol. per cent	vol. per cent	vol. per cent							
		vol.	per cent	vol.	per cent							vol.	per cent	vol.	per cent	vol.		
2	10.5	26.6	25.5	43.4	53.3	48.2	58.1	29.9	39.4	10.0	9.9	9.5	1.61	1.47	1.0	1.0		
14	18.7	41.2	42.0	42.2	53.9	49.4	61.8	31.8	42.9	11.7	12.4	11.1	1.55	1.44	1.1	1.1		
15	20.7	48.8	49.0	46.1	59.0	56.1	70.3	35.7	47.3	12.9	14.2	11.6	1.57	1.49	1.1	1.1		
10	23.7	55.6	54.9	36.6	50.5	44.4	60.9	30.4	42.0	13.9	16.5	11.6	1.46	1.45	1.2	1.4		
11	24.3	56.2	54.8	38.6	52.6	48.1	63.7	31.3	43.4	14.0	15.7	12.1	1.53	1.47	1.1	1.3		

*D* refers to the ratio of bicarbonate in cells to that in serum. The subscripts *TS*, *B*, and *C*, stand for true serum, blood and blood cells, respectively.

demonstrated in such a small number of experiments. The ratio of  $\Delta[\text{BHCO}_3]$  of true serum to  $\Delta[\text{BHCO}_3]$  of whole blood averages  $1.1 \pm 0.1$ , which agrees fairly well with the ratio found by Stadie *et al.* (8). This merely shows that  $\Delta[\text{BHCO}_3]$  is greater for true serum than for whole blood. It may be pointed out that this ratio 1.1 corresponds to that found for the ratio of the slopes of the carbon dioxide absorption curves of true serum and whole blood at a given temperature (9).

There is a general tendency for  $\Delta[\text{BHCO}_3]$  of cells and  $\Delta[\text{BHCO}_3]$  of serum to increase with increasing oxygen capacity. If  $\Delta[\text{BHCO}_3]$  of cells is plotted against oxygen capacity, an almost exact rectilinear relationship is noted. The agreement between  $\Delta[\text{BHCO}_3]$  of serum and oxygen capacity is much less exact. Hemoglobin concentration, then, affects  $\Delta[\text{BHCO}_3]$  of cells, and much of the load of increased bicarbonate at reduced temperature is borne by the cells. The lower serum/cell bicarbonate distribution coefficients ( $1/D$  in Table IV) at the lower temperature afford further indication that the cells carry the major portion of the extra bicarbonate.

There are two experiments in which true serum was separated at  $38^\circ$  and saturated at  $23^\circ$ , and true serum was separated at  $23^\circ$  and saturated at  $38^\circ$ . The respective values for  $\Delta$  in these experiments are 1.25 and 3.32 volumes per cent in a blood with oxygen capacity of 5.65 volumes per cent, and 4.95 and 4.32 volumes per cent in the blood with oxygen capacity of 24.3 volumes per cent. These four values have an average of 3.5 volumes per cent.  $\Delta[\text{BHCO}_3]$  of separated serum is much less than that of blood or true serum. These findings confirm those of Stadie *et al.* (8). Changes in bicarbonate naturally involve transfer of base from some other acids, of which protein is the most important. Therefore, changes of bicarbonate cannot be as great in separated serum, where only the serum proteins function, as in whole blood or true serum, where the buffer effects of hemoglobin are called into play. Serum proteins were not determined in these separated sera. Possibly they were less in the experiment with the lower oxygen capacity.

We have previously (29) noted that initial bicarbonate concentration directly affects the value of  $\Delta[\text{CO}_2]_{60-30 \text{ mm.}}$  in a given separated serum. If temperature effects are similar to tension effects,

it would be reasonable to expect that  $\Delta[\text{BHCO}_3]$  of the serum separated at 23° would exceed that of the 38° serum. However, this was observed in only one of the two separated sera studied. As has been emphasized by us before (29), "separated serum" is not an entity. Its reactions to given tension and presumably to given temperature changes are conditioned by its composition at the time

TABLE V  
*Effect of Temperature on CO<sub>2</sub> Absorption Curve*

	Temperature	Pressure	Experiment 14	Experiment 15		Temperature	Pressure	Experiment 14	Experiment 15
	°C.	mm.	vol. per cent	vol. per cent		°C.	mm.	vol. per cent	vol. per cent
Oxygen capacity Cell volume			18.7	20.7	$\Delta[\text{BHCO}_3]_{23-38^\circ}$ Blood		30	11.4	11.8
	38	30	41.2	48.1		40	11.7	12.9	
	38	60	42.8	49.4		60	11.8	14.2	
	23	30	40.2	48.6		30	12.4	13.9	
	23	60	42.2	49.2		40	12.4	14.2	
Bicarbonate content Blood					$\Delta[\text{BHCO}_3]_{30-60 \text{ mm.}}$ Blood		60	12.2	14.4
	38	30	38.0	42.2		38		10.8	10.1
	38	60	48.8	52.3		23		11.2	12.5
	23	30	49.4	54.0		38		11.1	12.9
	23	60	60.6	66.5		23		10.9	12.4
Serum	38	30	45.0	51.2	Serum				
	38	60	56.1	63.6					
	23	30	57.4	65.1					
	23	60	68.3	78.0					

the change is initiated. It is difficult to decide whether, in order to obtain the value of  $\Delta[\text{BHCO}_3]$  of separated serum, one should use an average of the values obtained from 23° and 38° sera or whether the serum should be separated at an intermediate temperature point and then saturated at 38° and at 23°. In view of the doubtful character of separated serum, it seems safer to extrapolate its value from the value for anemic blood specimens. As the result of all the experiments recorded in Table I,  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  of sepa-

rated serum would be calculated as 6.7 volumes per cent, the point in Fig. 1 where the line defining the relation of hemoglobin to  $\Delta[\text{BHCO}_3]$  cuts the  $y$  axis.

*CO<sub>2</sub> Absorption Curve*—Two 30 and 60 mm. CO<sub>2</sub> absorption curves were determined at 23° and 38°. The values for CO<sub>2</sub> capacity at 40 mm. were calculated from the 30 and 60 mm values. In each case the whole blood slope as expressed by  $\Delta[\text{CO}_2]_{60-30 \text{ mm.}}$  was greater at 23° than at 38°, while the serum slope was greater at 38° (Table V).

One notes a general tendency for  $\Delta[\text{BHCO}_3]_{22-38^\circ}$  to increase with increasing bicarbonate content, the first evidence obtained that the initial bicarbonate concentration influences the temperature effect. It may well be that just as initial bicarbonate concentration affects the response to a given tension change (29) in any one blood, so also does it influence the temperature effect in any one blood, while in a heterogenous collection of bloods or sera, the bicarbonate effect cannot be demonstrated or evaluated, because there are too many other variables. There are twelve experiments, which can be divided into six pairs of equal oxygen capacity. In four pairs,  $\Delta[\text{BHCO}_3]$  is greater in the specimen having the higher bicarbonate content at 38°, in the other two pairs  $\Delta[\text{BHCO}_3]$  is greater in the specimen having the lower bicarbonate content at 38°.

*pH and Cell Volume*—Before considering pH changes, it may be well to emphasize that, with changing temperature, pH and pOH are not reciprocally related as they are at any one temperature  $[\text{H}^+] \times [\text{OH}^-] = Kw = \text{constant}$ . Alteration in temperature may cause a simultaneous increase or decrease in both  $[\text{H}^+]$  and  $[\text{OH}^-]$ .

The pH of serum can be estimated only approximately from analyses of whole blood. It was calculated by means of the Henderson-Hasselbalch equation (4, 6) with 6.14 as  $\text{pK}_1$  at 38°. For temperatures other than 38°, Cullen, Keeler, and Robinson's (13) correction of 0.005 increase in  $\text{pK}_1$  for each degree of fall in temperature was used. The use of a constant  $\text{pK}_1$  for whole blood is of course improper (1), and the use of a constant correction for temperature may also be invalid. Since  $\Delta[\text{BHCO}_3]$  does not vary uniformly for each degree of temperature change, it is possible that the variation in  $\text{pK}_1$  is not uniform. Stadie and Martin (7) considered that for a pH range of as much as 1 unit, the assumption of

a constant  $pK_1$  for whole blood was sufficiently accurate for comparative purposes.  $pK_1$  of whole blood is not constantly related to  $pK_1$  of serum. The difference between the two is affected by hemoglobin content and pH (1). Since the pH range of these bloods is only about 0.1 unit, the effect of pH on  $pK_1$  is negligible. The variation of hemoglobin is, however, considerable and would probably change the value of  $pK_1$  to an appreciable extent. But Stadie and Martin (7) have pointed out that the factors changing  $pK_1$  at one temperature would likewise change it in the same direction at the other temperature. Since we have chosen a definite temperature interval, the effects of hemoglobin for any one blood would presumably alter  $pK_1$  to the same extent at both temperatures and should not affect the value of  $\Delta pH_{23-38^\circ}$ .

There seems to be no agreement among previous workers on the subject of the effect of temperature change on pH. In the series of whole bloods reported here, pH sometimes increases and sometimes decreases as the temperature falls.

Because of the discrepancies between results obtained in other laboratories and within our own series, it seems desirable to subject the data of Table I to further analysis. If the experiments are divided into hemoglobin content groups as they were for Fig. 1, certain conclusions can be drawn. In the first group of severe anemias, pH decreases with temperature. At oxygen capacity of 10 to 14 volumes per cent, pH is about the same at both temperatures. Above 14 volumes per cent of oxygen capacity, pH is higher at 23° than 38°, and the increment in pH tends to vary according to the hemoglobin content. Accordingly, the different results obtained by former investigators may be due to varying amounts of hemoglobin in the bloods studied. It may be objected that the previous reports concerned pH changes in serum rather than in whole blood. But since true serum partakes of the buffer system of the cells with which it has been in contact, its reactions are functions of the hemoglobin content of the whole blood from which it is derived.

The most extensive series of data relating serum pH to temperature change is offered by Stadie *et al.* (8). The magnitude of the pH change observed by them is the same for true or separated serum, in both dog and sheep blood. Experimentally they found that pH increases about 0.2 unit when temperature falls from

38–20°, bicarbonate remaining constant. By assuming as an approximation a linear relationship between  $[\text{BHCO}_3]$  and pH at both temperatures, they were able to calculate the pH changes with carbon dioxide tension constant. There is neither experimental nor theoretical proof of the linear relationship of  $[\text{BHCO}_3]$  to pH at both 20° and 38°. Under the limited conditions of Stadie and Martin's experiments, bicarbonate remaining constant, it follows that the base bound by hemoglobin cannot change appreciably. But data obtained under such restricted conditions cannot be used to calculate the effect on pH at constant  $\text{CO}_2$  tension, where much more profound changes must inevitably ensue. At any rate, their values averaged about 0.02 pH increase for a temperature decrease from 38–20° at constant  $\text{CO}_2$  tension. The change is of the same order of magnitude as that observed in our experiments on whole blood. In all but one instance their calculated values for pH increased with temperature fall. This circumstance may be due to the fact that none of their sera was derived from anemic blood.

The variations in whole blood  $\Delta\text{pH}_{23-38^\circ}$  noted by us are implicit in the Henderson-Hasselbalch equation from which pH is calculated and in the fact that  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  varies directly as the hemoglobin content. The increase in  $\text{H}_2\text{CO}_3$ , either from  $\text{CO}_2$  tension rise or temperature fall, is usually greater proportionally than the increase in bicarbonate. When  $\text{CO}_2$  tension is increased, temperature remaining constant, pH must decrease because  $\text{pK}_1$  is a constant. In bloods of high hemoglobin content  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  is relatively large, while  $\Delta[\text{H}_2\text{CO}_3]$  is relatively small; also  $\text{pK}_1$  increases with temperature fall. The net result is that  $\Delta\text{pH}_{23-38^\circ}$  is a positive quantity. In severe anemias,  $\Delta[\text{BHCO}_3]_{23-38^\circ}$  is relatively small, while  $\Delta[\text{H}_2\text{CO}_3]$  is relatively large. The increment in  $\text{pK}_1$  is not enough to offset the value of the fraction,  $\Delta(\log[\text{BHCO}_3] - \log [\text{H}_2\text{CO}_3])_{23-38^\circ}$ , and  $\Delta\text{pH}_{23-38^\circ}$  becomes a negative quantity. It follows that at some intermediate hemoglobin points  $\Delta\text{pH}_{23-38^\circ} = 0$ , and there is no pH change.

Temperature change does not consistently affect cell volume. The hematocrit value at 23° is sometimes higher than that at 38°. Less often it is lower. The percentage differences vary from –8 to +9 and are not apparently correlated with the pH changes.

However, if the data are once again subjected to statistical treatment according to the four groups of hemoglobin content used in Fig. 1, a tendency for cell volumes to be greater at the lower temperature may be noted in the group of bloods with low hemoglobin. This corresponds with the zone of decreased pH. The other three groups, in order of ascending oxygen capacities, show decreasing average hematocrit changes, so that in the last group where  $\Delta\text{pH}_{23-38^\circ}$  is strongly positive,  $\Delta(\text{cell volume})_{23-38^\circ}$  is slightly negative.

Were cell volume merely a function of pH, we should expect that where  $\Delta\text{pH}_{23-38^\circ} = 0$ ,  $\Delta(\text{cell volume})_{23-38^\circ}$  would = 0. It has been pointed out above that the calculation of pH for whole blood is but an approximation and can be used only for comparative purposes. Therefore, even the establishment of qualitative and directional relationships between  $\Delta\text{pH}$  and hemoglobin and between cell volume and  $\Delta\text{pH}$  must be of significance.

*Comparison of Tension and Temperature Effects*—Both rise of CO<sub>2</sub> tension and fall of temperature increase physically dissolved CO<sub>2</sub>; a concomitant increase of bicarbonate is an obligatory result. It remains to be seen whether tension and temperature effects are similar quantitatively as well as qualitatively. In other words, does the concentration of H<sub>2</sub>CO<sub>3</sub> which produces a certain amount of bicarbonate at 23° produce the same amount at 38°? Haggard (16) claimed this to be true of dog whole blood, and Van Slyke and Cullen (15) stated that it was true in human separated serum.

On the other hand, the experiments reported here would lead to the conclusion that the changes observed in response to temperature are not even of the same nature as those resulting from change of CO<sub>2</sub> tension. The decrease in temperature necessary to produce a certain increment of H<sub>2</sub>CO<sub>3</sub> has a quantitatively different effect on bicarbonate than results when an equal increment of H<sub>2</sub>CO<sub>3</sub> is produced by increasing CO<sub>2</sub> tension. For example, in average whole blood the difference in dissolved CO<sub>2</sub> at 38° and 23° is about (3.8 - 2.6) volumes per cent. If 2.6 volumes per cent of H<sub>2</sub>CO<sub>3</sub> are dissolved at 40 mm., then 3.8 volumes per cent are dissolved at 58 mm. at the same temperature. In other words the temperature change from 38° to 23° is approximately equivalent, as far as H<sub>2</sub>CO<sub>3</sub> is concerned, to a tension change from 40 to 58 mm. But changing the temperature at 40

mm. from 38° to 23° results in an increase of bicarbonate of about 14 volumes per cent, while changing the tension from 40 to 60 mm. at 38° increases the bicarbonate only about 7 volumes per cent. The same considerations apply to a smaller extent to separated serum. Changes of tension and temperature which are equivalent as far as  $\text{H}_2\text{CO}_3$  is concerned cause entirely different changes of bicarbonate, the effect of temperature being greater.

The magnitude of the effect that a given temperature change will have on any determinant of the carbon dioxide absorption curve of blood is a function of the hemoglobin content of the blood. Hemoglobin acts as an acid both at body and at room temperature and consequently competes with bicarbonate for base. Elevation of temperature increases the dissociation constants of both hemoglobin acid and carbonic acid, but its effect on the former is much the greater. Hence at 38° hemoglobin binds more base and leaves less for the formation of bicarbonate. At room temperature there is an excess of physically dissolved  $\text{CO}_2$  over that at body temperature, and the extra  $\text{CO}_2$  is at hand to combine with the base released by hemoglobin.

Another factor which may influence the amount of base bound by hemoglobin is the shift of the isoelectric point with temperature change. Stadie, Austin, and Robinson (8) calculated that pH and the isoelectric point were changed to the same extent by a given temperature change, bicarbonate remaining constant. Under such circumstances the change in isoelectric point would not affect the base-binding power of hemoglobin. But Stadie and Martin (7) and Warburg (12) state that pH is about 0.3 unit more acid at body temperature than 18–20° (the conditions of the experiments are not presented). If this is true at constant  $\text{CO}_2$  tensions, then the increment in isoelectric point is so much greater than the pH increase that a considerable change in the base-binding power of hemoglobin must result.

#### SUMMARY

The effects of decreasing the temperature of blood while  $\text{CO}_2$  tension is maintained constant are:

1. An increase in carbonic acid and bicarbonate. This increase is not uniform for each degree of fall in temperature. It is greater around 23° than around 38°.

2. The increase in bicarbonate for the temperature fall from 38–23° is directly proportional to the hemoglobin content of the blood. An equation expressing this relationship was derived from the statistical results of thirty-five experiments and has the form  $\Delta[\text{BHCO}_3]_{23-38} = (0.32 \text{ oxygen capacity} + 6.7) \text{ volumes per cent}$ . By means of this equation the bicarbonate content at 38° can be calculated from the bicarbonate content at 23°.

3. The effect of temperature on the CO<sub>2</sub> capacity of true serum is 1.1 times its effect on the corresponding whole blood.

4. The effect of temperature on the pH of whole blood depends on the hemoglobin content of the latter. Very anemic bloods show a decreased pH, while normal bloods show an increased pH at the lower temperature.

5. Tension and temperature effects are dissimilar. A tension increase of about 30 mm. causes about the same increment of H<sub>2</sub>CO<sub>3</sub> as does a temperature decrease from 38–23°. This increment of H<sub>2</sub>CO<sub>3</sub> from temperature change has about twice as great an effect on bicarbonate as does the same increment of H<sub>2</sub>CO<sub>3</sub> resulting from an increase of CO<sub>2</sub> tension at constant temperature.

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## SULFUR IN PROTEINS

### V. THE EFFECT OF ALKALIES UPON CYSTINE, WITH SPECIAL REFERENCE TO THE ACTION OF SODIUM HYDROXIDE\*

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Cystine is commonly believed to be exceedingly unstable in the presence of alkalies; however, the mechanism of its decomposition by alkalies and the nature of the products which are formed are very imperfectly known. Among those who have contributed to the study of this problem in recent years are Hoffman and Gortner (1), Hoffman (2), Bergmann and Stather (3), Bergmann, Andrews, and Andrews (4), Brand and Sandberg (5), Plimmer and Lowndes (6), Andrews (7-9), Gortner and Sinclair (10), Clarke and Inouye (11, 12), Nicolet (13), and Daft and Coghill (14). Deaminization, elimination of sulfur from the molecule, racemization, and reduction to cysteine are known to occur. Oxalic acid, pyruvic acid, and *i*-alanine have been identified as decomposition products. Bergmann and Stather (3) postulate that the primary reaction of alkalies on cystine is the formation of  $\alpha$ -aminoacrylic acid and sodium sulfide, that the  $\alpha$ -aminoacrylic acid then by hydrolysis yields pyruvic acid and ammonia, by reduction yields alanine, and by oxidation yields oxalic acid. Nicolet (13) suggests that the  $\alpha$ -aminoacrylic acid arises by an enolization of the carboxylic carbonyl group followed by a 1:4 elimination of hydrogen sulfide. Gortner and Sinclair (10) found that solutions of the hydroxides

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of barium, calcium, and strontium, and even dilute solutions of sodium carbonate were much more effective in removing nitrogen and sulfur from the cystine molecule than were strong (20 per cent) solutions of sodium or potassium hydroxides. None of the postulated mechanisms of decomposition accounts for such differences in the behavior of the various alkalies as Gortner and Sinclair observed. It was with the hope of elucidating some of the factors which might be involved that the present study was undertaken.

#### EXPERIMENTAL

*Methods*—Unless otherwise stated, 2.5 gm. of cystine and 50 cc. of the alkaline solution to be studied were placed in a 125 cc. Pyrex Erlenmeyer flask which was immediately attached to a reflux condenser and set on a sand bath kept at such a temperature that boiling began in approximately 10 minutes and proceeded at a moderate rate. At the end of the desired time interval, reckoned from 10 minutes after the flask was placed on the sand, the flask was raised and its contents cooled somewhat by pouring about 50 cc. of wash water through the condenser.

The alkaline solution was immediately washed into a 1 liter Claissen flask with about 100 cc. of water and kept under reduced pressure of about 30 mm. at 45–55° for 15 minutes in order to remove ammonia. After the 15 minutes HCl was added to the residual solution to give a final acid concentration of about 0.1 N HCl in the final 250 cc. volume. The mixture was again placed under diminished pressure at 45–55° for another 15 minutes to remove hydrogen sulfide or any other volatile sulfur compounds which may have been liberated by the acid.

Any precipitated colloidal sulfur or silica in the residue was removed by filtration and the washings of this precipitate were added to the filtrate. The filtrate and washings were collected in a 250 cc. volumetric flask and made up to volume with water. This solution, which in almost all cases was perfectly clear, was kept in a stoppered Erlenmeyer flask until the various analyses had been completed. For convenience, this solution will be referred to as the "hydrolysate."

Total nitrogen was determined by the Kjeldahl method applied to 25 cc. aliquots of the hydrolysate.

Amino nitrogen was determined by means of the Van Slyke micro apparatus with 2 cc. aliquots of a 1:1 aqueous dilution of the hydrolysate. Observed values were multiplied by the factor 0.901 to correct for the abnormal behavior of cystine toward nitrous acid. While this factor does not agree with the value of 0.926 given by Van Slyke (15), it represents our value obtained from repeated determinations with pure cystine at 25° under conditions similar to those used for the unknown mixtures.

Total sulfur was determined on 25 cc. aliquots of the hydrolysate by the Benedict-Denis method as described by Hoffman and Gortner (16).

Residual optical activity was measured by direct observations on the hydrolysate. Errors due to the relatively low concentration of HCl were largely eliminated by calculating the per cent of residual activity with respect to some of the pure cystine treated similarly except for being acidified immediately after its solution in the alkali.

Cystine was determined by Sullivan's method according to the directions given in one of his latest papers (17). The cystine-containing solution was prepared by diluting from 5 to 25 cc. of the hydrolysate to a volume of 100 cc. with water, the amount of hydrolysate taken being such that on the basis of sulfur and nitrogen analyses it should give a reading close to that of the standard. In the case of solutions containing barium, this element was removed from the final dilution by adding sodium sulfate before making to volume, followed by subsequent filtration.

The method of study differs from previous work, particularly in the removal of liberated ammonia and hydrogen sulfide by evacuation rather than by means of a current of air or inert gas passing out through a cooled reflux condenser. The method also differs from earlier work, except that of Gortner and Sinclair (10) in the determination of *residual* nitrogen, sulfur, etc., rather than that *liberated* in some particular form. It is believed that this procedure reflects more directly the fate of the cystine itself since the results are practically independent of secondary changes which certain of the decomposition products may undergo. This would be especially true with reference to the state of oxidation of the sulfur after its removal from the molecule because sulfides, polysulfides, sulfites, thiosulfates, and colloidal sulfur, which might

be present under various conditions, all would be removed as volatile hydrogen sulfide and sulfur dioxide or insoluble free sulfur.

When sulfide alone is determined, it may represent only a fraction of the sulfur originally liberated from the cystine. The hydrolysates obtained in the present work never contained more than traces of sulfates, which would be included in the residual sulfur.

*Materials*—The cystine used in this study showed the following analysis on an ash- and moisture-free basis (ash 0.15 per cent, moisture 0.73 per cent at 105°): total N 11.55 per cent (theory 11.65 per cent), total S 26.22 per cent (theory 26.69 per cent),  $\text{NH}_2\text{-N}$  (corrected for factor 0.901) 11.77 per cent (theory 11.65 per cent),  $[\alpha]_D^{25} = -206.2^\circ$  (accepted value  $-211.2^\circ$ ). Specific rotation was determined under the conditions specified by Andrews (7) and the theoretical value was calculated from the formula of Toennies and Lavine (18). The material was free from tyrosine. The alkalis and other reagents were of the usual research grade.

*Experimental Data*—The analytical data are expressed as per cent of the various elements, groups, or properties remaining in the mixture after the treatment which is indicated. The theoretical values for pure cystine are taken as the basis for calculation in the case of sulfur and nitrogen analyses. In the case of optical activity and cystine determinations, the calculations are based on the values obtained with the original cystine under similar conditions of measurement.

The figures listed for total nitrogen, amino nitrogen, and total sulfur are in every case the average of at least two figures agreeing closely with each other. The acceptable difference between these two figures was never more than 2 per cent of their average value, except for five or six cases of relatively low residual concentration, and was usually much less than this.

The degree of accuracy with which the whole experimental procedure can be reproduced is illustrated by the two sets of values for the action of 4 N NaOH during a 12 hour heating period and the two for a 24 hour period (cf. Table I).

*Action of Potassium Hydroxide on Cystine*—A 4 N solution of potassium hydroxide was used, the decomposition time intervals chosen being 0, 1, 4, 12, and 24 hours. For zero time the cystine was merely dissolved in the KOH solution and immediately acidified without heating. As the solutions increased in temperature

to the boiling point, they changed rapidly from colorless to yellow and then to red which slowly became more brownish red. The final acidified hydrolysates were orange-red in color except for the unheated solution which was colorless. The data obtained are shown in Table I.

*Action of Sodium Hydroxide on Cystine*—The concentration of NaOH, time intervals, and the procedure were exactly similar to

TABLE I  
*Rate of Decomposition of L-Cystine by Various Hydroxides at Boiling Temperature*

Alkali used	Time of heating	Residual				
		Total N	Amino N	Total S	Cystine	Optical activity
	hrs.	per cent	per cent	per cent	per cent	per cent
4 N KOH	0	98.1	99.9	98.7	100	100
	1	82.3	78.6	83.9	77	49
	4	79.8	75.5	80.5	72	46
	12	77.1	74.3	75.5	61	35
	24	73.7	73.0	70.9	57	31
4 N NaOH	0	98.0	99.0	97.4	100	100
	1	81.7	79.0	81.8	74	33
	4	77.9	76.2	77.0	66	28
	12	75.3	72.9	72.7	52	18
	12	74.3	72.3	73.5	55	20
	24	70.5	69.2	66.4	39	13
	24	71.8	71.2	68.3	47	13
4 N Ba(OH) <sub>2</sub>	0	97.5	100.1	97.2	100	100
	1	35.1	38.9	28.6	12	3
	4	27.0	34.9	13.7	8	0
	12	23.4	31.5	15.6	1	0
	24	22.4	30.4	16.8	1	0

those in the case of KOH. Color changes during heating of the alkaline solution were like those produced by KOH but the final acidified hydrolysates were yellow to orange in color rather than orange-red. The data are given in Table I.

Curves plotted from the data of Table I for potassium hydroxide and sodium hydroxide are very similar both in type and sequence. Moreover, the two curves, except for optical activity, are practically superimposable. It therefore appears that NaOH and KOH

do not differ significantly in their ability to decompose *l*-cystine. This is in agreement with results recently reported by Andrews (9) and contrary to the earlier observations of Gortner and Sinclair (10). It is obvious that NaOH and KOH do not effect anywhere near a complete removal of sulfur from cystine. We have been unable to confirm the experiment on which Gortner and Sinclair (10) based their belief that this might occur.

The very close agreement between the rates of removal of nitrogen and sulfur indicates that serine is not formed in the reaction or that if it is formed it must decompose at least as fast as the sulfur is removed from the cystine molecule. Accordingly, no attempt was made to isolate serine from these decomposition mixtures.

Perhaps the most surprising of the results, and the most difficult of explanation, are the relatively small amount of decomposition and especially the rapidly decreasing rate of reaction. The decomposition starts out rapidly and then slows down so sharply that after 24 hours it has practically stopped, although analyses indicate that the mixture still contains 70 per cent of the substance undergoing decomposition. A possible explanation of the mechanism involved will be considered later.

*Action of Barium Hydroxide on Cystine*—Although the effect of saturated aqueous barium hydroxide upon cystine had been previously studied by Gortner and Sinclair (10), it was thought desirable to study its effect at a concentration as nearly equivalent to 4 N as was conveniently possible. Since a solution of this strength cannot be prepared at room temperature, 31.5 gm. of  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  were placed in each flask, then 35 cc. of water, and finally the 2.5 gm. of cystine. At the boiling temperature the hydroxide is easily soluble in the water which is present. These mixtures were subjected to the same procedure as in the NaOH and KOH experiments.

In all cases, the mixtures became pale yellow soon after boiling began and remained so during the entire heating period. The behavior of this system differed also from the previous ones by an apparent pronounced evolution of gas during the decomposition, perhaps in part the result of a finely divided precipitate dispersed throughout the boiling liquid. During the early stages, this precipitate was very likely made up in part of the normal barium

salt of cystine (19). In contrast to the experiments with KOH and NaOH, acidification of the alkaline mixture gave very little precipitate and the final hydrolysates were clear yellow in color with no indication of orange or brown. The smaller amount of elemental sulfur produced in this case agrees with the observation of Gortner and Sinclair (10) that  $\text{Ba}(\text{OH})_2$  changes a much larger proportion of the cystine sulfur into the volatile hydrogen sulfide than does  $\text{Na}_2\text{CO}_3$ . The analytical results are summarized in Table I.

The results, with  $\text{Ba}(\text{OH})_2$ , agree with previous observations of Gortner and Sinclair (10) and of Andrews (9) in that this alkali causes much greater decomposition of cystine than do NaOH and KOH. The form of the curves obtained by plotting the data indicates that the reaction in this case is not essentially different from the others but that it goes nearly to completion. An amino nitrogen content higher than the total nitrogen is of course impossible and the consistently higher values found in this series of data are possibly due to side reactions between the relatively large proportion of decomposition products and the nitrous acid. Cystine itself requires a correction factor in the Van Slyke analysis, the factor varying somewhat with the conditions employed, and it has been observed that whenever the amount of decomposition is great, the agreement between total and amino nitrogen is less satisfactory than when only a small amount of decomposition has taken place.

With  $\text{Ba}(\text{OH})_2$ , as well as with the other alkalis, the rate of racemization of cystine is considerably greater than is the rate of decomposition, regardless of whether the latter is measured by the Sullivan reaction or by nitrogen and sulfur analyses. Andrews (8) had previously obtained somewhat similar results in studying the decomposition of cystine by NaOH at room temperature in a nitrogen atmosphere.

*Effect of Concentration of Alkali on Cystine Decomposition*—Although Andrews (8, 9) has already reported rather complete studies of the relationship between concentration of alkali and deamination of cystine, it was thought advisable to study the effect of NaOH solutions, with the conditions and methods of the present work which also include sulfur elimination, cystine concentration, and optical activity.

A series of cystine-NaOH solutions was prepared, ranging from the normal sodium salt of cystine to 1 cystine:19.2 NaOH. The analyses were carried out as in the previous experiments, the time of heating being 12 hours in all cases.

The data in Table II confirm Andrews' (8) observation concerning the deaminization of cystine by varying concentrations of NaOH and show that sulfur elimination, decrease in amino nitrogen, and destruction of cystine are all affected in a similar manner, although at different rates for the lower concentrations of alkali.

While a complete study of the effect of concentration of  $\text{Ba}(\text{OH})_2$  was not made, Table II shows that the effect of this alkali is just

TABLE II  
*Effect of Concentration of Alkali on Decomposition of l-Cystine by Aqueous Solutions at Their Boiling Temperatures (Time, 12 Hours)*

	Alkali per mol cystine	Residual				
		Total N	Amino N	Total S	Cystine	Optical activity
	<i>mols</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
NaOH	2	61.1	45.2	60.8	32	18
	4	67.2	57.3	67.7	52	21
	6	68.7	63.4	66.7	61	13
	10	71.8	67.2	70.1	69	13
	19.2	74.8	72.6	73.1		19
$\text{Ba}(\text{OH})_2$	1	57.0	43.8	54.3	17	13
	9.6	23.4	31.5	15.6	1	0

opposite to that of NaOH. However, when NaOH and  $\text{Ba}(\text{OH})_2$  are present in amounts just sufficient to form the normal salts of cystine (2 mols of NaOH and 1 mol of  $\text{Ba}(\text{OH})_2$  per mol of cystine), the rate and extent of decomposition are almost identical. Again these results are in agreement with the data of Andrews (9).

In order to be able to follow the changes more satisfactorily, a separate study was made of the rate of fall in optical activity of cystine in several alkaline solutions kept in stoppered flasks at room temperature. Readings were made on aliquots after acidifying with sufficient hydrochloric acid and diluting with water to give a solution containing as nearly 1 gm. of cystine per 100 cc. of normal acid as possible. The solutions used were: Solution A, 5 gm. of cystine in 200 cc. with NaOH equivalent to the cystine;

Solution B, 5 gm. of cystine in 200 cc. with NaOH to give excess alkali concentration of 0.1 N; Solution C, 5 gm. of cystine in 200 cc. of Ba(OH)<sub>2</sub> equivalent to the cystine; Solution D, 5 gm. of cystine in 200 cc. with Ba(OH)<sub>2</sub> to give excess alkali concentration

TABLE III

*Rate of Fall in Optical Activity of l-Cystine in Varying Concentrations of NaOH and Ba(OH)<sub>2</sub> at Room Temperature (5 Gm. Cystine in 200 Cc. Volume)*

The readings are given in degrees Ventzke.

Time	NaOH				Ba(OH) <sub>2</sub>			
	Normal salt (Solution A)		Normal salt in 0.1 N NaOH (Solution B)		Normal salt (Solution C)		Normal salt in 0.1 N Ba(OH) <sub>2</sub> (Solution D)	
	Reading	Per cent of initial activity	Reading	Per cent of initial activity	Reading	Per cent of initial activity	Reading	Per cent of initial activity
<i>hrs.</i>								
0	-11.7	100	-11.6	100	-11.5	100	-11.5	100
24	-11.2	96	-11.2	96	-11.4	99	-11.2	97
119	-11.4	97	-11.5	99	-11.4	99	-6.1	53
192	-11.2	96	-10.6	91	-11.0	96	-5.0	43
729	-8.4	72	-6.8	59	-8.6	75	-3.5	30

TABLE IV

*Rate of Fall in Optical Activity of l-Cystine in 4 N NaOH at Room Temperature (5 Gm. Cystine in 100 Cc.)*

Time	Reading	Per cent of initial activity
<i>hrs.</i>	<i>degrees Ventzke</i>	
0	-10.9	100
1	-9.6	88
2	-9.3	85
3	-8.5	78
7	-7.8	71
24	-4.5	41

of 0.1 N; Solution E, 5 gm. of cystine in 100 cc. of 4 N NaOH. The data are shown in Tables III and IV. The rate of fall in optical activity of *l*-cystine increases very decidedly with increasing alkali concentration, not only in the case of Ba(OH)<sub>2</sub> but also with NaOH. Apparently the processes of *racemization* and of

*decomposition* of cystine respond differently to changes in concentration of NaOH, while they respond similarly in Ba(OH)<sub>2</sub> solutions. This is one of several indications that the decomposition of cystine by NaOH is abnormally complicated by side reactions tending toward increasing stability of the cystine.

*Mechanism of Decomposition of Cystine by Sodium Hydroxide*—Although the data in Table I indicate no great fundamental differences in the reaction mechanism during the early stages of the decomposition of cystine by KOH, NaOH, and Ba(OH)<sub>2</sub>, respectively, they do require some special explanation in the case of NaOH and KOH for the early stopping of the reaction. Several possibilities suggested themselves and had to be considered: (1) Cystine might be completely decomposed but with formation of unusual nitrogen and sulfur products not removed by the procedure followed in this work; (2) natural cystine might be a mixture of two or more isomeric forms of which at least one, occurring in large proportion, is stable toward NaOH and KOH; (3) progress of the reaction might depend not only on the presence of the alkali but also on some other factor which under the conditions employed was limited in its operation; (4) the reaction might be stopped merely by mass action effect of accumulated reaction products, an effect which in the case of the more active alkalies might be eliminated by removal of at least one product in insoluble form; (5) side reactions might occur which interfere with the normal decomposition process, perhaps a case of combination of decomposition products with unchanged cystine, resulting in the stabilization of the latter.

The first of these is highly improbable, because the analyses by Sullivan's method indicate that a large proportion of the original cystine is still present. Neither is the second hypothesis very probable, since several workers have found that the optical isomers decompose in a similar manner. Furthermore, if this alone were the correct explanation, then the concentration of alkali should not greatly affect the end result and different alkalies should behave more nearly alike.

In order definitely to eliminate these two possible hypotheses of the surprisingly small amount of decomposition caused by NaOH and KOH, it was necessary to isolate the residual cystine and to show that it would again decompose in the same manner

as the original cystine. This has been done in the case of cystine boiled for 12 hours with 4 N NaOH.

*Residual Cystine from NaOH Hydrolysate*—It had been observed that when the HCl-acidified hydrolysates from the action of NaOH on cystine were evaporated to dryness *in vacuo*, cystine could be extracted, presumably in the form of the hydrochloride, from the dry salt mass with hot 95 per cent ethyl alcohol. Four 2.5 gm. portions of cystine were boiled with 50 cc. portions of 4 N NaOH for 12 hours and the residual solutions subjected to the usual procedures for the removal of volatile decomposition products. The final acidified solutions were combined, filtered, evaporated to dryness *in vacuo*, and the salt residue extracted repeatedly with hot ethyl alcohol containing a little water until the volume of

TABLE V  
*Properties of Residual Cystine after Boiling for 12 Hours with 4 N NaOH, As Compared with Original l-Cystine*

Constituent or property	Residual cystine	l-Cystine
Total N, per cent.....	10.94	11.55
Amino " " ".....	12.15	11.77
Total S, " " ".....	25.76	26.22
Specific rotation at 26°, degrees.....	-112	-206.2
Solubility in water, at 30°, gm. per 100 cc. ....	0.0500	0.0096

extract totaled about 500 cc. After addition of 300 cc. of water the alcoholic solution was made alkaline with ammonium hydroxide, then acidified slightly with acetic acid. After standing for 24 hours, the light brown precipitate which resulted was filtered off, dissolved in dilute HCl, decolorized with a small amount of norit, and filtered. After the addition of about one-third of a volume of alcohol, the filtrate was made alkaline with ammonium hydroxide, acidified with acetic acid, and allowed to stand for about a day. The precipitate was filtered off, washed thoroughly with water, then with alcohol, and finally with ether. The dried product was white and granular and amounted to 4.05 gm. or 40.5 per cent of the original cystine.

Microscopic observation showed the product to be made up of three types of crystals: some perfect hexagonal plates, a larger number of needles frequently arranged in stellate groups, and a

large proportion of imperfect spheres. Analyses and physical properties of this material are given in Table V in comparison with those for the original *l*-cystine.

2.5 gm. of this residual cystine were boiled with 50 cc. of 4 N NaOH for 12 hours and treated in the usual manner. This mixture behaved exactly as did the original *l*-cystine and the figures given below show that it gave practically the same analytical results. Obviously then, the small extent of decomposition of *l*-cystine by NaOH cannot be due to the presence in natural cystine of some isomeric form possessing greater stability toward alkalis.

Substance	Residual		
	Total N	Amino N	Total S
	per cent	per cent	per cent
<i>l</i> -Cystine.....	74.8	72.6	73.1
Residual cystine.....	73.0	76.4	74.9

There can be no doubt that the product isolated was cystine which had escaped decomposition by the NaOH, the only important effect of the alkali being partially to racemize the cystine. This isolation of residual cystine in a 40 per cent yield confirms the analytical data already presented. Many of the statements in the literature concerning the extreme instability of cystine in alkaline solutions, particularly of the caustic alkalis, are certainly in error.

*Effect of Added Decomposition Products and Other Substances on Alkali Decomposition of Cystine*—With the first two possibilities definitely eliminated, it remained to study the other three in greater detail. In order to do this, a series of decompositions was run in the usual manner with 4 N NaOH and a heating period of 12 hours but with slight modifications consisting chiefly in the addition of cystine decomposition products and related compounds. Special conditions and the results are indicated very briefly in Table VI. In all cases, added material was included in the total volume of 50 cc. by taking 25 cc. of 8 N NaOH, making the additions, and diluting to volume.

The greater stability of cysteine toward the alkali as compared with cystine is in accord with the observations of Andrews (9) and Clarke and Inouye (11). The high value for amino nitrogen

in this case may be due to the inapplicability of the cystine correction factor to cysteine. In view of the facts that preliminary reduction of the cystine to cysteine did not cause more complete stability than was observed and that the presence of excess air did not increase the extent of decomposition which was found under normal conditions, it seems rather improbable that the incomplete-

TABLE VI

*Effect of Added Substances on Decomposition of 2.5 Gm. of L-Cystine by 50 Cc. of 4 N NaOH at Its Boiling Temperature (Time, 12 Hours)*

Added substance (or conditions)	Residual				
	Total N	Amino N	Total S	Cystine	Optical activity
	per cent	per cent	per cent	per cent	per cent
None.....	74.8	72.6	73.1	53	19
Cystine reduced to cysteine with tin and HCl before decomposition.....	88.3	94.5	85.3	57	0
Air stream, 250 cc. per min. over surface and out of reflux.....	74.1	70.5	75.3	55	31
Na <sub>2</sub> CO <sub>3</sub> (0.4 gm.).....	74.8	75.7	74.1	53	25
“ (0.8 “ ).....	72.3	75.6	74.0	53	27
NH <sub>4</sub> OH (2.8 cc., 28 per cent), Na <sub>2</sub> S·9H <sub>2</sub> O (2.5 gm.), S (0.34 gm.), pyruvic acid (1.84 gm.), all equivalent to 2.5 gm. cystine.					
No cystine in this mixture.....	20.1	8.0	6.4	0	0
NH <sub>4</sub> OH (0.8 cc., 28 per cent).....	74.3	72.0	73.4	53	22
Na <sub>2</sub> S·9H <sub>2</sub> O (2.5 gm.).....	75.1	75.4	65.8		8
“ (1.25 “ ) + S (0.17 gm.).....	71.0	68.9	63.0	52	20
Pyruvic acid (0.91 gm.).....	67.7	60.5	62.1	54	11
Oxalic “ (1.26 “ ).....		78.1	79.1		
Formic “ (1.36 “ Na salt)....		74.5	73.2		
Formaldehyde (10 cc., 40 per cent).	81.5	67.2	85.8	40	17

ness of the decomposition of cystine by NaOH can be due simply to reduction of the cystine to the more stable cysteine. Since the presence of excess air and of carbonate had no influence on the normal course of reaction, it appears that neither of these factors is essential and that the extent of decomposition of cystine under the present conditions could not have been limited by an insufficient supply of oxygen or carbon dioxide.

The mixture of primary decomposition products of cystine was run through the usual procedure merely to see what would result if cystine did decompose completely and rapidly according to the reaction suggested by Bergmann and if the properties of the resulting mixture depended on the recombination of products. Obviously, any tendency toward such recombination was slight and the reaction mixture was quite different from those obtained in starting with cystine and NaOH. The values resembled more nearly those found for the cystine-Ba(OH)<sub>2</sub> system, indicating that perhaps the latter gives a simpler reaction than do the caustic alkalies.

That the cessation of decomposition of cystine in the NaOH is not due to a mere accumulation of a primary product which might be removed in the cases of the more active alkalies is evidenced by the results of the addition of ammonia, the sodium sulfides, and pyruvic acid. None of these retarded the decomposition at all, while pyruvic acid showed the accelerating catalytic action reported by Clarke and Inouye (11).

The studies which have been described in the preceding pages suggested that the relatively small amount of decomposition caused by strong NaOH was due to the stabilization of much of the cystine through side reactions involving secondary products of the decomposition which did occur. It was with this in mind that the effect of added oxalic acid was tried, and formic acid was added because of its close relationship to oxalic. Although these data are incomplete, they are sufficient to indicate that oxalic acid had a slight retarding effect on the rate of decomposition which formic acid did not exhibit.

The addition of formaldehyde was made with the idea of possibly stabilizing the nitrogen portion of the molecule which might permit a selective removal of the sulfur but it is evident that this did not occur. With a similar purpose in mind, 2.5 gm. of cystine were subjected to the action of 50 cc. of approximately 4 M sodium ethylate in absolute alcohol instead of 50 cc. of 4 M NaOH in water. The reflux condenser was closed with a calcium chloride tube and other conditions were similar to those of the experiments reported in Table VI. Water was not added until the acidification stage for removal of hydrogen sulfide. It was thought that

if the first stage of cystine decomposition involved elimination of hydrogen disulfide and the second removal of ammonia by hydrolysis, then by carrying out the reaction in an anhydrous medium the loss of nitrogen should be prevented or at least retarded. Actually this was not observed, possibly because hydrolysis might have been replaced by alcoholysis, but the extent of decomposition was found to be surprisingly high. Values for residual total nitrogen, amino nitrogen, total sulfur, cystine, and optical activity were, respectively, 23.9, 15.0, 20.5, 7, and 3 per cent. Apparently the stabilizing mechanism suggested as being set up in aqueous NaOH does not operate in the alcoholic solution.

*Ether- and Alcohol-Insoluble Fraction Described by Gortner and Sinclair*—The ether- and alcohol-insoluble fraction prepared by Gortner and Sinclair (10) from the decomposition products of 50 gm. of cystine in saturated  $\text{Ba}(\text{OH})_2$  and described by them as "extremely hygroscopic. . . . insoluble in nearly all organic solvents and so extremely soluble in water as to preclude the possibility of recrystallization" was still available for study. On examining it, it was obvious that the properties of this material had changed very considerably during the year or more since its preparation, because it was no longer noticeably hygroscopic and was only partially soluble in water.

Cystine was determined in this material by Sullivan's method and a content of 26 per cent was obtained. By dissolving some of the substance in dilute HCl, making alkaline with ammonia, acidifying with acetic acid, adding half a volume of alcohol, and allowing the mixture to stand several days, crystals were obtained which were insoluble in water and alcohol. On microscopic observation, these appeared to have the form of needles arranged singly and in stellate groups. All tests showed that they were crystals of inactive cystine. Unquestionably the ether- and alcohol-insoluble fraction obtained by Gortner and Sinclair contained a fair proportion of cystine, probably in its reduced form, cysteine, which, in the presence of other decomposition products, made a hygroscopic, water-soluble mass. On long standing in contact with air the cysteine, and possibly other thio compounds, was oxidized to the disulfide, decreasing the solubility in water and the hygroscopicity.

## DISCUSSION

Gortner and Sinclair (10) noted that practically all of the organic nitrogen remaining after the alkaline decomposition of cystine was still in the  $\alpha$ -amino form. Our findings confirm this conclusion. Our data also show that almost invariably the values for residual nitrogen and residual sulfur are in rather close agreement to a 1:1 ratio. Similar results have been reported by Clarke and Inouye (11) for the decomposition of cystine by  $\text{Ca}(\text{OH})_2$  and  $\text{NaOH}$  at room temperatures. These two facts are the expected consequences of the mechanism for the alkaline decomposition of cystine advocated by Bergmann and Stather (3) and should therefore be considered as further evidence in its favor. As far as the present discussion is concerned, it is immaterial whether the reaction involves direct elimination of  $\text{H}_2\text{S}_2$  between the  $\alpha$ - and  $\beta$ -carbon atoms as originally pictured by Bergmann or whether it is a case of 1-4 elimination of the same substance between the enolized carboxyl groups and the disulfide linkage as recently suggested by Nicolet (13). Nevertheless, the latter view is favored as Nicolet has already pointed out, by the fact that the rate of racemization of cystine by alkali is greater than is its rate of decomposition. This behavior was first reported by Andrews (8) and has been observed throughout our study.

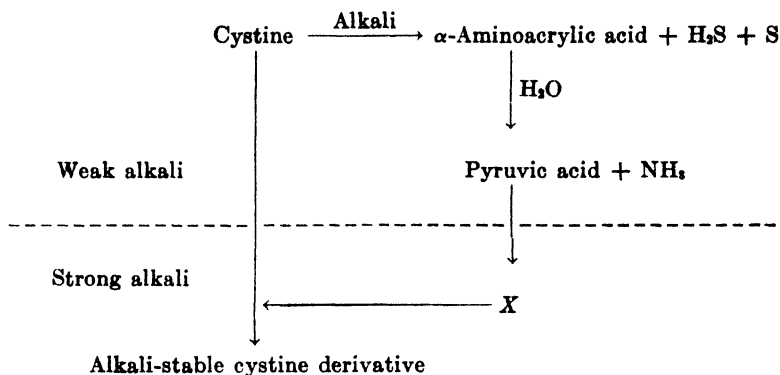
The similarity in type of decomposition curves obtained with  $\text{KOH}$ ,  $\text{NaOH}$ , and  $\text{Ba}(\text{OH})_2$  points toward the operation of the same primary reaction in spite of the great differences in the amount of change produced. It seems quite probable that the views of Bergmann and his coworkers and of Nicolet correctly represent the course of this primary reaction. They do not, however, without further development provide any explanation of many apparent irregularities which appear in the cystine literature. For example, it is difficult to see why the decomposition of cystine by  $\text{NaOH}$  and  $\text{KOH}$  should virtually stop when the reaction is only about a third completed, if it is as simple as these workers suggest. That the reaction does stop has been proved by analysis and by isolation of the residual cystine which in turn has been found to decompose in exactly the same manner as the original material.

In addition, there is the effect of concentration of  $\text{NaOH}$  and of  $\text{KOH}$  on the extent of decomposition caused by these alkalies.

One should expect the stronger alkali to cause more rapid removal of both sulfur and nitrogen. This has been found to be the case for all alkalies studied except for NaOH and KOH which behave in exactly the opposite way, at least above a relatively low concentration.

Finally, the erratic differences in the extent of action of various alkalies on cystine and in the effect of various added ions on the action of NaOH are in need of explanation. Attempts to do this on the basis of the ability of ions to remove liberated sulfide have given only discouraging results.

As a possible explanation of some of these apparent discrepancies we present the hypothesis of the stabilization of cystine by some specific secondary decomposition product. In that event, the decomposition of cystine could be represented diagrammatically as follows:



X being some secondary decomposition product capable of forming, under suitable conditions, a stable compound with undecomposed cystine. The stabilizing reaction would not take place if either pyruvic acid or X should be removed from solution or if the alkalinity were insufficient to permit the formation of X or its combination with the cystine. The catalytic accelerating action of pyruvic acid would be manifested in the case of weak alkalies which would not cause the usual polymerization or condensation.

It is quite obvious that if the type of reaction system pictured above were in operation it could lead to the rapidly decreasing rate of decomposition observed with strong NaOH or KOH solutions. As the reactions progress, the constantly increasing pro-

duction of  $X$  would stabilize an increasing proportion of cystine and leave a decreasing amount susceptible to decomposition until (assuming 1 mol of  $X$  per mol of cystine) the stabilized cystine should equal that already destroyed. The point where this should occur would, of course, depend on the relative rates of the two reactions under the conditions employed.

The concentration of the alkali should also be considered in relation to the two different but simultaneously occurring reactions. It is to be expected that increasing concentrations of alkali should increase the rate of the primary decomposition and it is not improbable that it should have a similar effect on the stabilizing or inhibiting reaction as a whole. Then, in any particular case, the observed effect of a change in alkali concentration would be the resultant of the effects on the two reactions acting in opposite directions. The data already presented, as well as the work of Andrews, show that for NaOH and KOH the quantity of cystine decomposed becomes considerably less as the concentration of alkali is increased above that approximately necessary for salt formation. This indicates quite definitely that the stabilizing reaction, whatever its nature, is greatly favored in the stronger alkali and perhaps even depends on a hydroxyl ion concentration above a certain minimum for its operation. For example, when NaOH and Ba(OH)<sub>2</sub> are used in concentrations just sufficient to give the normal cystine salt, there is practically no difference in their action and no appreciable stabilization. For alkalis, such as Ba(OH)<sub>2</sub>, Ca(OH)<sub>2</sub>, and Na<sub>2</sub>CO<sub>3</sub>, which in increasing concentrations or amounts cause increasing cystine destruction, it is necessary to assume that effects of concentration are being reflected largely in the primary reaction due to conditions unfavorable to the stabilization of cystine. Such conditions would be either low alkalinity or removal (or destruction) of substance  $X$  or a precursor, probably as an insoluble salt.

With the reaction system postulated above, the wide variations in extent of decomposition found for different alkalies and the effects of added metallic salts would find a logical explanation in the behavior of these substances toward the substance  $X$ . For example, if  $X$  formed salts with solubilities similar to those of the oxalates, then the great efficiency of the alkaline earth hydroxides in deaminizing cystine would not be difficult to understand. How-

ever, any conclusions in this direction must await further knowledge concerning the exact nature of the reaction or reactions by which cystine is rendered alkali-stable in strong NaOH and KOH solutions.

In the experimental work, color changes were observed in the various alkaline treatments of cystine. This difference in color produced may be of some significance. With NaOH and KOH the color changes produced by heating were from colorless to yellow to deep red and finally to brownish red, while with  $\text{Ba}(\text{OH})_2$  the mixtures remained yellow during the entire period. The colors observed are usually explained as being due to the formation of polysulfides and, in that case, the absence of red color in  $\text{Ba}(\text{OH})_2$  mixtures, as well as of others where decomposition is great, could possibly be the result of reduction of polysulfide to monosulfide by the larger amounts of organic reducing substances which should be present. Addition of reducing substances to NaOH containing cystine usually prevented the appearance of the deep red color. On the other hand, the fact that the rate of formation of this red color in NaOH decreased rapidly with decreasing concentration of alkali indicates that the color observed may bear some relationship to the stabilization of cystine.

#### SUMMARY AND CONCLUSIONS

A study has been made of the decomposition of *l*-cystine by aqueous KOH, NaOH, and  $\text{Ba}(\text{OH})_2$ . Special attention has been given to the mechanism of the reaction in the case of NaOH. The data lead to the following conclusions which confirm or agree with observations recently published by others.

1. All the alkaline mixtures which were studied removed nitrogen from the cystine molecule at very nearly the same rate as sulfur.

2. Practically all of the nitrogen remaining in solution after the decomposition of cystine by alkalis is in a form which can be determined by the Van Slyke method for  $\alpha$ -amino nitrogen.

3. Cysteine is definitely more stable toward boiling 4 N NaOH than is cystine.

4. Increasing concentrations of NaOH cause *decreasing* cystine destruction as measured by residual nitrogen, sulfur, and cystine determined according to Sullivan's method.

5. The fall in optical activity of *l*-cystine in alkaline solutions is considerably more rapid than is the decomposition of cystine.

In addition, the following conclusions seem justifiable.

6. 4 *N* NaOH and 4 *N* KOH both act on cystine to practically the same extent as regards nitrogen and sulfur elimination and cystine destruction.

7. The small extent of deaminization of cystine by NaOH and KOH which has been reported by several workers is due to an actual stability of the cystine and not to the formation of peculiar products under these conditions. This has been proved both by analysis and by the isolation of the crystalline cystine in 40 per cent yield from cystine which had been boiled for 12 hours in 4 *M* NaOH solution.

8. The relatively great stability of cystine toward NaOH (and presumably also KOH) appears to be the result of a secondary reaction between some decomposition product and undecomposed cystine leading to stabilization of the latter. This is indicated by the type of reaction curves obtained and by the fact that the isolated residual cystine was found to decompose in exactly the same manner as the original *l*-cystine. Such a stabilization hypothesis is presented.

9. It is probable that the ether- and alcohol-insoluble fraction, obtained by Gortner and Sinclair (10) by boiling cystine for 24 hours with saturated Ba(OH)<sub>2</sub>, consisted of cysteine mixed with non-nitrogenous products, since a sample after standing for somewhat over a year had changed markedly in physical properties and was found to contain a fair proportion of *i*-cystine.

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## STUDIES ON RACEMIZATION

### XII. ACTION OF ALKALI ON POLYPEPTIDES COMPOSED OF LEVO-ALANINE

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The present communication deals in the first place with the problem of the effect of the number of constituent amino acids of a peptide on the degree of racemization through the action of dilute alkali. In order to avoid the effect of differences in structure of the amino acids, polypeptides were prepared composed of alanine only. Di-, tri-, tetra-, and pentapeptides were used for the experiments. These peptides were allowed to stand at 25° for periods of 2, 8, and 16 days, the concentration of alkali being 0.2 and 1.0 N and the proportion of alkali to peptide being 2:1 and 10:1. The results of these experiments are given in Table I. In Column 4 is given the racemization of the amino acids of each peptide calculated on the basis of the total number of amino acids entering into the structure of the peptide, and in Column 5 the average racemization of racemizable amino acid calculated on the basis of the total number of amino acids less the two terminal ones, inasmuch as it may be accepted that these two are not susceptible to racemization.

It is evident that the differences in the degree of racemization calculated by one or the other method must differ with the length of the chain of the peptides. Referring to the degree of racemization calculated on the basis of the total number of amino acids as the over-all racemization, and to that calculated on the total number less the two terminal ones as the true racemization, the ratio between the first and the second values will increase with the increase of the length of the chain. In fact, by this ratio it is possible to establish the length of the chain. In the case of

proteins, the ratio may be regarded equal to unity in view of the length of the chain.

From the data reported in Table I, it may be concluded that the rates of racemization of the amino acids in the tetra- and penta-

TABLE I  
*Summary of Racemization of Polypeptides Composed of Levo-Alanine by Sodium Hydroxide at 25°*

Substance	Concentration of NaOH		Duration	Total racemization	Average racemization of racemisable amino acid
	(1)	Equivalent to substance (2)			
	<i>N</i>		<i>days</i>	<i>per cent</i>	<i>per cent</i>
1. Levo-alanyl-levo-alanine	0.2	2	2	0	
	0.2	2	8	0	
	0.2	2	16	0	
	1.0	10	2	4 (?)	
	1.0	10	8	4 (?)	
	1.0	10	16	4 (?)	
2. Di-levo-alanyl-levo-alanine	0.2	2	2	4	12
	0.2	2	8	9	27
	0.2	2	16	11	33
	1.0	10	2	8	24
	1.0	10	8	19	57
	1.0	10	16	19	57
3. Tri-levo-alanyl-levo-alanine	0.2	2	2	6	12
	0.2	2	8	21	42
	0.2	2	16	21	42
	1.0	10	2	22	44
	1.0	10	8	32	64
	1.0	10	16	32	64
4. Tetra-levo-alanyl-levo-alanine	0.2	2	2	8	13
	0.2	2	8	23	38
	0.2	2	16	29	48
	1.0	10	2	24	40
	1.0	10	8	37	61.5
	1.0	10	16	37	61.5

peptides are of the same order of magnitude. In the tripeptide it is markedly lower in the experiments with 0.2 *N* alkali and less marked in the experiments with higher concentration; in the latter, in the 8 and 16 day experiments, the values are not much different from those of the higher peptides. That the rate of racemization

of a tripeptide should be lower than that of the higher peptides is to be expected in view of the fact that as soon as one amino acid is hydrolyzed off, the remaining dipeptide is no longer racemizable under the conditions of the experiment.

In Table II are given data previously published on racemization of several other tri- and tetrapeptides containing one or two leucine residues susceptible to racemization. Again it is seen that the degree of racemization of the tripeptides is lower than that of the tetrapeptides. Furthermore, it is seen that the degree of racemization of the tripeptides is of the same order of magnitude whether

TABLE II

*Summary of Previous Work Done on Racemization of Polypeptides by Sodium Hydroxide at 25° for 8 Days*

Substance	Concentration of NaOH	[ $\alpha$ ] <sub>D</sub> <sup>20</sup> of amino acid in hydrolysate with 20 per cent HCl (+NaCl)		Total racemization after 8 days	Average racemization of racemizable amino acid
		Racemized	Control		
	<i>N</i>	<i>degrees</i>	<i>degrees</i>	<i>per cent</i>	<i>per cent</i>
1. Dextro-leucyl-dextro-leucyl-dextro-leucine	0.2	-15.5	15.5	0	0
	1.0	-14.9		4	12
2. Levo-alanyl-dextro-leucyl-dextro-leucine	0.2	-14.0	-13.9	0	0
	1.0	-13.2		5	13.5
3. Dextro-leucyl-dextro-leucyl-glycyl-glycine	1.0	-13.0	16.1	19	38
4. Glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine	1.0	-12.3	16.1	23.5	35

both terminal components are leucine or whether one is leucine and the other alanine. Also in the two tetrapeptides differing in their composition but both having leucine as the racemizable component, the degree of racemization under identical conditions is of the same order of magnitude.

On comparing the data given in Tables I and II, it may be seen that the degree of racemization of peptides composed of alanine is higher than that of those composed of leucine and yet the stability of leucine peptides is greater than that of those composed of alanine. *The conclusion thus may be arrived at that the amino acids*

*which confer stability on a peptide with respect to hydrolytic agents do the same with respect to racemization.* Thus, taking alanine as the parent substance of all other optically active aliphatic  $\alpha$ -amino acids (which may be regarded as derived from alanine by the substitution of the methyl group by other radicles, or by the substitution of the 2nd hydrogen atom by a second alkyl group), it was found that peptides composed of alanine alone were the least stable, and those of the type of isovaline were the most stable. All other peptides composed of amino acids containing a hydrogen atom on carbon atom (2) but differing from alanine occupied in the scale of stability a position intermediate between the two first named.<sup>1</sup> The same relationship has been observed with regard to ketopiperazines. *Thus, the relative degree of racemization of polypeptides higher than tripeptides seems to depend principally on the nature of the amino acids, all external conditions being the same.* It is realized that the experimental observations need to be extended before this conclusion is firmly established.

But even the limited data available at this date are of considerable significance for the explanation of the observations made on the racemization of proteins. It was found that the action of alkalis on proteins was different from that on ketopiperazines. In the latter case dilute alkalis (0.1 or 0.2 N) produced extensive racemization of not less than about 70 per cent,<sup>2</sup> whereas 1.0 N alkali caused hydrolysis of the ketopiperazine to the dipeptide without racemization of the latter. On the other hand, proteins showed a degree of racemization higher with the stronger alkali than with the more dilute.<sup>3</sup> Another peculiarity in regard to racemization of proteins was the fact that under identical conditions individual proteins suffered different degrees of racemization. Are these peculiarities of the behavior of proteins consistent with the theory of the polypeptide structure?

The experiments on the alanine peptides reported in this communication clearly show that, similarly to proteins, they are racemized to a higher degree with 1.0 N alkali than with 0.2 N. Furthermore, the degree of racemization attained in 8 days by the

<sup>1</sup> Levene, P. A., Steiger, R. E., and Rothen, A., *J. Biol. Chem.*, **97**, 719 (1932).

<sup>2</sup> Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **86**, 703 (1930).

<sup>3</sup> Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **82**, 171 (1929).

racemizable components of these peptides is of the same order of magnitude as that of the amino acids of albumin, fibrin, and gelatin. The degree of racemization of edestin and casein under the same conditions is somewhat higher, but this difference may be explained on the assumption that there are amino acids which racemize at a higher rate than alanine.

Summarizing from the data reported in this communication it follows:

1. The degree of racemization of polypeptides under identical conditions is dependent upon the structure of the amino acid.

2. In polypeptides higher than tripeptides the degree of racemization of the racemizable amino acids seems to be independent of the number of constituent amino acids.

3. Under identical conditions, the course of racemization of individual amino acids in polypeptides is similar to that in proteins.

4. The progress of racemization of proteins is consistent with the theory of the polypeptide structure and not with the theory of the ketopiperazine structure.

It is realized that thus far these conclusions are based on limited experimental material. Unfortunately, the preparation of sufficient quantities of the higher peptides is beset by many difficulties. An important question which has not yet been sufficiently investigated is the mutual effect of neighboring amino acids with respect to racemization.

## EXPERIMENTAL

### *Preparation of Material*

1. *Dextro- and Levo-Alanine*—(a) Dextro-alanine was obtained by hydrolysis of silk fibroin. The hydrochlorides of the esters were converted into the free esters by the barium hydroxide and chloroform method. It was found advantageous to initiate the reaction by the addition of a small quantity of 40 per cent sodium hydroxide to the ester hydrochlorides. The esters obtained from 1 kilo of silk fibroin were worked up in one operation. The reaction mixture was kept in an ice-water bath during the entire operation.

The alanine obtained in this manner had the following rotation.

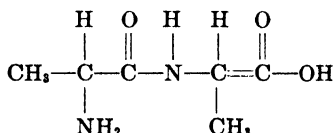
$$[\alpha]_D^{25} = \frac{+ 1.15^\circ \times 5}{2 \times 0.2006} = + 14.33^\circ \text{ (in 20 per cent hydrochloric acid)}$$

(b) *Levo*-alanine was prepared by aminating *levo*-bromopropionic acid which was obtained from *d*-alanine. The rotation of the *levo*-bromopropionic acid thus prepared ranged within  $-43^\circ$  to  $-45^\circ$  and that of the *levo*-alanine was as follows:

$$[\alpha]_D^{25} = \frac{-1.24^\circ \times 5}{2 \times 0.2136} = -14.51^\circ \text{ (in 20 per cent hydrochloric acid)}$$

2. *Levo-Bromopropionyl Chloride*—*Levo*-bromopropionyl chloride was prepared in the usual way by means of thionyl chloride. The rotation of the substance (homogeneous) was between  $-36^\circ$  and  $-38^\circ$ .

### 3. *Levo-Alanyl-Levo-Alanine*



(a) *Levo-Bromopropionyl-Levo-Alanine*—30 gm. of freshly prepared *levo*-bromopropionyl chloride were condensed in the usual way with 12.5 gm. of *levo*-alanine. The yield was 20 gm. or 64 per cent of the theory. The crude product had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.50^\circ \times 5}{2 \times 0.0845} = +14.79^\circ \text{ (in water)}$$

(b) *Amination*—10 gm. of the crude *levo*-bromopropionyl-*levo*-alanine were readily dissolved in 111 cc. of concentrated ammonium hydroxide (sp. gr. 0.90). The solution was allowed to stand with occasional shaking at room temperature for 3 days. It was then evaporated under reduced pressure to dryness. A little absolute alcohol was added and the solution again evaporated to dryness. The dry residue was refluxed with 100 cc. of methyl alcohol for about 30 minutes to remove the ammonium bromide formed. After cooling, the refluxed mixture was filtered and washed with a little alcohol. For purification the crude dipeptide was dissolved in twice its weight of boiling water. Absolute alcohol (200 cc.) was added to the solution, which became milky immediately. On cooling, the dipeptide crystallized in glistening plates. The

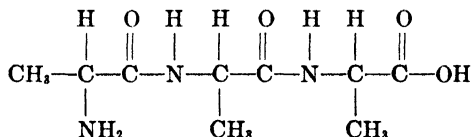
crystals were filtered off, washed with a little alcohol, and dried in a vacuum oven at 50°. The yield was 5.5 gm. or 74 per cent of the theory. This dipeptide had the following rotations and composition.

$$[\alpha]_D^{20} = \frac{+ 1.02^\circ \times 5}{2 \times 0.1169} = + 21.8^\circ \text{ (in water)}$$

$$[\alpha]_D^{20} = \frac{+ 0.73^\circ \times 5}{2 \times 0.05} = + 36.5^\circ \text{ (in 2 N hydrochloric acid)}$$

4.681 mg. substance: 7.739 mg. CO<sub>2</sub> and 3.125 mg. H<sub>2</sub>O  
 4.800 " " : 0.74 cc. N at 24° and 749 mm.  
 20.20 " " : 3.18 " " " 19° " 737 "  
 C<sub>8</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>. Calculated. C 44.97, H 7.55, N 17.50, NH<sub>2</sub> 8.75  
 160.09 Found. " 45.08, " 7.47, " 17.46, " 8.72

#### 4. Di-Levo-Alanyl-Levo-Alanine



(a) *Levo-Bromopropionyl-Levo-Alanyl-Levo-Alanine*—40 gm. of levo-bromopropionyl chloride were condensed in the usual way with 20 gm. of the dipeptide. The levo-bromopropionyl-levo-alanyl-levo-alanine was obtained as a thick suspension which did not settle after standing overnight in the cold. It was filtered by suction, washed thoroughly with ice water and a little alcohol, and then dried in a vacuum oven at 50°. The yield of crude product was 30 gm. or 83 per cent of the theory.

(b) *Amination*—30 gm. of the crude levo-bromopropionyl-levo-alanyl-levo-alanine were treated as in the case of levo-bromopropionyl-levo-alanine. The yield was 20.5 gm. or 87 per cent of the theory. The tripeptide showed a bluish pink biuret reaction, and had the following rotation and composition.

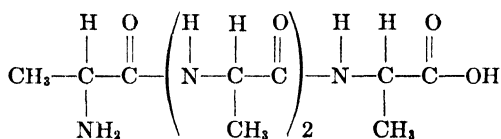
$$[\alpha]_D^{20} = \frac{+ 1.55^\circ \times 5}{2 \times 0.05} = + 77.5^\circ \text{ (in 2 N hydrochloric acid)}$$

Abderhalden<sup>4</sup> reported the corresponding tripeptide of the dextro series to have the following rotation.

$$[\alpha]_D^{25} = -72.2^\circ \text{ (in 2 N hydrochloric acid)}$$

4.046 mg. substance:	7.696 mg. CO <sub>2</sub> and 2.750 mg. H <sub>2</sub> O
4.090 " "	: 0.623 cc. N at 25° and 763 mm.
18.40 " "	: 1.94 " " " 24 6° " 762 "
(C <sub>9</sub> H <sub>17</sub> N <sub>3</sub> O <sub>4</sub> ) + $\frac{1}{2}$ H <sub>2</sub> O.	Calculated. C 44.98, H 7.56, N 17.50, NH <sub>2</sub> 6.10
240.10	Found. " 45.13, " 7.60, " 17.51, " 5.87

### 5. Tri-Levo-Alanyl-Levo-Alanine



(a) *Levo-Bromopropionyl-Di-Levo-Alanyl-Levo-Alanine*—18 gm. of levo-bromopropionyl chloride were condensed in the usual way with 17.5 gm. of di-levo-alanyl-levo-alanine. The crude product weighed 22.5 gm. or 80 per cent of the theory.

(b) *Amination*—10 gm. of finely powdered levo-bromopropionyl-di-levo-alanyl-levo-alanine were dissolved in 165 cc. of concentrated ammonium hydroxide (sp. gr. 0.90). It formed a cloudy solution after shaking for some time. After standing at room temperature for 3 days, the solution was evaporated to dryness under reduced pressure. The evaporation was repeated by the addition of about 50 cc. of absolute alcohol. The residue was refluxed with 150 cc. of methyl alcohol for about 30 minutes. After cooling, the refluxed mixture was filtered and washed with a little absolute alcohol. For purification the crude tetrapeptide was suspended in 150 cc. of boiling water. On adding 2 cc. of concentrated ammonium hydroxide, it dissolved. The solution was shaken with about 5 gm. of norit and filtered. To the filtrate 500 cc. of boiling absolute alcohol were added. On cooling and gentle shaking, the tetrapeptide crystallized in glistening plates. After standing overnight in the cold, it was filtered, washed with a little alcohol, and dried in a vacuum oven at 50–60°. The yield was 6.50 gm. or 74 per cent of the theory. The tetrapeptide is very

<sup>4</sup> Abderhalden, E., *Fermentforschung*, **13**, 55 (1931).

insoluble in water and gives a purplish biuret reaction. It has the following rotation and composition.

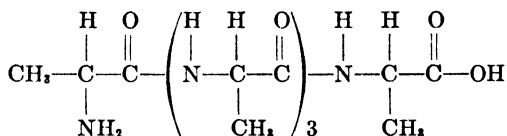
$$[\alpha]_D^{25} = \frac{+2.30^\circ \times 5}{2 \times 0.05} = +115^\circ \text{ (in 2 N hydrochloric acid)}$$

The corresponding tetrapeptide of the dextro series was reported by Abderhalden<sup>4</sup> to contain 1 molecule of water and to have the following rotation.

$$[\alpha]_D^{25} = -120.5^\circ \text{ (in 2 N hydrochloric acid)}$$

4.480 mg. substance	7.530 mg. CO <sub>2</sub> and 3.060 mg. H <sub>2</sub> O
4.365 " "	0.701 cc. N at 25° and 753 4 mm.
19.00 " "	1.56 " " " 23.5° " 754 "
(C <sub>12</sub> H <sub>22</sub> N <sub>4</sub> O <sub>5</sub> ) + $\frac{1}{2}$ H <sub>2</sub> O. Calculated.	C 46.27, H 7.77, N 17.99, NH <sub>2</sub> 4.49
311.18 Found.	" 45.83, " 7.64, " 18.30, " 4.54;
	ash, none

#### 6. Tetra-Levo-Alanyl-Levo-Alanine



(a) *Levo-Bromopropionyl-Tri-Levo-Alanyl-Levo-Alanine*—To a solution of 14.4 gm. of tri-levo-alanyl-levo-alanine in 92 cc. of 0.5 N sodium hydroxide, kept below 0° in an ice-salt mixture, were added 15.5 gm. of levo-bromopropionyl chloride and 200 cc. of 0.5 N sodium hydroxide alternately in small portions with vigorous shaking. During the process of treatment, which occupied about 60 minutes, the temperature was never allowed to rise above 0°. Gelatinous levo-bromopropionyl-tri-levo-alanyl-levo-alanine separated. When the coupling was complete, 30 cc. of 5 N hydrochloric acid were added together with 100 cc. of water to make the reaction mixture fluid. After standing overnight in the cold, it was filtered and washed thoroughly with water. The residue was voluminous when moist but shrank to a hard small mass after drying in a vacuum oven at 50°. The crude product weighed 16.3 gm. or 83 per cent of the theory.

(b) *Amination*—16.3 gm. of the finely powdered levo-bromo-

propionyl-tri-levo-alanyl-levo-alanine were suspended in 300 cc. of concentrated ammonium hydroxide (sp. gr. 0.90) by shaking mechanically for about an hour. After standing for 4 days at room temperature, the suspension appeared crystalline and was evaporated under reduced pressure to dryness. 50 cc. of absolute alcohol were added and the evaporation repeated. The dry residue was refluxed with 250 cc. of methyl alcohol for about half an hour. After cooling, the refluxed mixture was filtered, washed with a little methyl alcohol, and dried. The crude pentapeptide weighed 14 gm. or 96 per cent of the theory.

For purification 7 gm. of the crude pentapeptide were suspended in 340 cc. of boiling water. It dissolved on adding 4 cc. of concentrated ammonium hydroxide. The solution was shaken with about 5 gm. of norit and filtered. To the filtrate were added 700 cc. of boiling absolute alcohol. On heating the alcoholic solution on a steam bath for about 30 minutes to drive off the ammonia, the pentapeptide separated as an amorphous powder. It was filtered off, washed with a little alcohol, and dried in a vacuum oven at 50–60°. The pure pentapeptide weighed 5.3 gm. and gave a purplish biuret reaction. It had the following rotation and composition.

$$[\alpha]_D^{25} = \frac{+2.65^\circ \times 5}{2 \times 0.05} = +132.5^\circ \text{ (in 2 N hydrochloric acid)}$$

The corresponding pentapeptide of the dextro series was reported by Abderhalden<sup>4</sup> to have the following rotation.

$$[\alpha]_D^{18} = -136.4^\circ \text{ (in 2 N hydrochloric acid)}$$

4.141 mg. substance:	7.024 mg. CO <sub>2</sub> and 2.760 mg. H <sub>2</sub> O
4.955 " "	: 0.787 cc. N at 29° and 760 mm.
26.00 " "	: 1.705 " " " 23.5° " 754 "
8.480 " "	: 0.025 mg. ash
(C <sub>11</sub> H <sub>27</sub> N <sub>5</sub> O <sub>6</sub> ) + H <sub>2</sub> O.	Calculated. C 45.98, H 7.46, N 17.89, NH <sub>2</sub> 3.60
391.22	Found. " 46.25, " 7.45, " 17.94, " 3.54

*Racemization with Sodium Hydroxide at 25° of Polypeptides Composed of Levo-Alanine*

Samples of peptides were weighed into Pyrex test-tubes (18 × 150 mm.) and dissolved in 7 cc. of standard alkali (0.2 and 1.0 N) and allowed to stand for periods of 2, 8, and 16 days. At the

end of each period 7 cc. of concentrated hydrochloric acid were added. The tubes were then sealed and heated at 125–130° for 6 hours in a glycerol bath. Optical rotations were made on the hydrolysate in 4 dm. tubes.

For analysis, 5 cc. in the experiment with the di- and tripeptide, and 3 cc. with the tetra- and pentapeptides were withdrawn,

TABLE III

*Racemization of Levo-Alanyl-Levo-Alanine and of Di-Levo-Alanyl-Levo-Alanine by Sodium Hydroxide at 25°*

	Time	After acid hydrolysis at 125-130°				Total racemisation
		Total N per cc.	$\alpha_D^t$ 4 dm. tube	$t$	$[\alpha]_D^t$	
Levo-alanyl-levo-alanine						
	<i>days</i>	<i>mg.</i>	<i>degrees</i>	<i>°C.</i>	<i>degrees</i>	<i>per cent</i>
Racemization by 0.2 N NaOH	Control	1.381	-0.48	26	-13.68	
	2	1.381	-0.48	28	-13.68	0
	8	1.381	-0.48	27	-13.68	0
	16	1.381	-0.48	28	-13.68	0
Racemization by 1.0 N NaOH	Control	1.381	-0.48	26	-13.68	
	2	1.381	-0.46	28	-13.11	4 (?)
	8	1.381	-0.46	27	-13.11	4 (?)
	16	1.381	-0.46	28	-13.11	4 (?)
Di-levo-alanyl-levo-alanine						
Racemization by 0.2 N NaOH	Control	2.071	-0.72	27	-13.67	
	2	2.071	-0.69	25	-13.10	4
	8	2.071	-0.65	27	-12.53	9
	16	2.071	-0.64	28	-12.34	11
Racemization by 1.0 N NaOH	Control	2.071	-0.72	26	-13.67	
	2	2.071	-0.66	27	-12.53	8
	8	2.071	-0.58	27	-11.20	19
	16	2.071	-0.58	28	-11.20	19

neutralized to phenolphthalein with 5 N sodium hydroxide, and diluted to 15 cc. Total nitrogen (Kjeldahl) and amino nitrogen (micro-Van Slyke, 15 minutes shaking) were made on 10 cc. and 2 cc. respectively.

Control experiments were carried out in each instance in the same manner as described for the racemization, except that the substance was dissolved in 7 cc. of concentrated hydrochloric acid

to which were then added the 7 cc. of standard alkali and the solution heated at 125–130° for 6 hours.

The weights of each peptide taken were as follows:

1. Levo-alanyl-levo-alanine 0.0007 mol = 0.1121 gm.
2. Di-levo-alanyl-levo-alanine 0.0007 " = 0.1681 "
3. Tri-levo-alanyl-levo-alanine 0.0007 " = 0.2178 "
4. Tetra-levo-alanyl-levo-alanine 0.0007 " = 0.2738 "

TABLE IV

*Racemization of Tri-Levo-Alanyl-Levo-Alanine and of Tetra-Levo-Alanyl-Levo-Alanine by Sodium Hydroxide at 25°*

	Time	After acid hydrolysis at 125-130°				Total racemization
		Total N per cc.	$\alpha_D^t$ 4 dm. tube	$t$	$[\alpha]_D^t$	
Tri-levo-alanyl-levo-alanine						
	days	mg.	degrees	°C.	degrees	per cent
Racemization by 0.2 N NaOH	Control	2.807	-0.96	27	-13.45	
	2	2.807	-0.90	26	-12.61	6
	8	2.807	-0.76	27	-10.65	21
	16	2.807	-0.76	28	-10.65	21
Racemization by 1.0 N NaOH	Control	2.807	-0.96	27	-13.45	
	2	2.807	-0.75	26	-10.51	22
	8	2.807	-0.65	27	-9.10	32
	16	2.807	-0.65	28	-9.10	32
Tetra-levo-alanyl-levo-alanine						
Racemization by 0.2 N NaOH	Control	3.451	-1.20	27	-13.67	
	2	3.451	-1.10	26	-12.53	8
	8	3.451	-0.92	27	-10.48	23
	16	3.451	-0.85	28	-9.68	29
Racemization by 1.0 N NaOH	Control	3.451	-1.20	27	-13.67	
	2	3.451	-0.91	26	-10.37	24
	8	3.451	-0.75	27	-8.57	37
	16	3.451	-0.75	28	-8.57	37

The degree of total racemization was calculated from the following formula.

$$\frac{[\alpha_0]_D^t - [\alpha_1]_D^t}{[\alpha_0]_D^t} \times 100 = \text{per cent}$$

where  $[\alpha_0]_D^t$  is the specific rotation of the control experiments and  $[\alpha_1]_D^t$  the specific rotation of the racemized amino acids. The obtained values are recorded in Tables III and IV.

# COMPARATIVE STUDIES ON THE ADSORPTION BEHAVIOR OF CRUDE VITAMIN A, CAROTENE, AND CHOLESTEROL

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## INTRODUCTION

Work in this laboratory on the adsorption behavior of vitamin A and related substances was initiated in 1929-30 when two of us (H. N. H., V. G. L.) attempted the concentration of this vitamin from the non-saponifiable portion of cod liver oil. Almost from the first we directed our efforts to adsorption methods of separating vitamin A in order to minimize oxidation or other undesired chemical reactions.

Little has been published on the adsorption of vitamin A. Kobayashi and Yamamoto (1) observed a color reaction with Japanese acid clays, Florida clays, and fullers' earth in contact with cod liver oil or solutions of vitamin A in volatile solvents. Lachat, Dutcher, and Honeywell (2) stated that vitamin A in cod liver oil may be adsorbed on highly activated silica gel so tenaciously that it cannot be rendered available when the silica gel is fed to rats. Toluene, they observed, removes the adsorbed vitamin A from the silica gel, while acetone extracts are inactive. Still more recently von Euler and Karrer (3) made reference to the use of fibrous alumina as an adsorbent in concentration studies on this vitamin.

It should be thoroughly understood that mere removal of vitamin A from a solvent is not necessarily due to adsorption, but may result from chemical destruction or from both. Effective adsorption implies the possibility of later liberating or recovering the vitamin from the porous solid used.

Willstätter has made most effective use of various forms of alumina in concentrating enzymes from water systems, and various

workers have used clays and similar materials in concentrating enzymes and even the water-soluble vitamins from water. However, it is a very different matter to adsorb and recover the fat-soluble vitamins; new difficulties, such as the problem of evaporating fats, present themselves.

Throughout our 1st year of work, vigorous efforts were made to improve the adsorption technique. In all of the work the Norris and Church (4) modification of the Carr-Price antimony trichloride reaction was used in estimating the amount of vitamin A present. It was felt that this chemical determination was sufficiently specific and accurate for the work since cod liver oil is known to contain the active chromogen, and since the determinations were in all cases a comparison of the intensity of color produced by different amounts of the same chromogen.

An important step in the study was the adsorption of vitamin A from petroleum ether and chloroform solutions of cod liver oil by silica gel and norit (Fig. 1). Petroleum ether proved to be the better solvent from which to adsorb, while norit showed greater removal of the vitamin. However, this combination proved to be unfortunate for recovery.

Solutions of the non-saponifiable fraction of cod liver oil offered more satisfactory material for these studies since so much of the extraneous material could be removed prior to adsorption. The cold saponification method of Marcus (5) was used to obtain this concentrate.

A new technique was here developed, early in 1930, based on the different adsorption results obtained with different solvents. The material was adsorbed from a solvent which permitted good adsorption and recovered, or liberated, by use of a better solvent from which there was poor adsorption. With this earlier work as the foundation, more extensive experiments have been carried out in the last 2 years.

### *Materials*

*Cod Liver Oil*—In the most recent experiments, material which had been obtained by nearly complete removal of cholesterol from a Squibb concentrate of the non-saponifiable fraction, and by distillation of this residue under 2 to 3 mm. pressure, at 176–196°, was used in petroleum ether solution.

*Carotene*—Preliminary experiments exhausted the supply of carotene prepared in this laboratory by the methods of Holmes and Leicester (6). In the work represented by Fig. 3 the carotene used was a pure product of The British Drug Houses Ltd., presented to us by Dr. Ellice McDonald, of the Cancer Research Laboratories, University of Pennsylvania.

*Cholesterol*—The cholesterol was a pure product prepared by the Eastman Kodak Company.

*Solvents*—The solvents used were dried over anhydrous calcium chloride and filtered just before use. In the most recent experiments, special care was taken to redistil the solvents in a stream of carbon dioxide or nitrogen, since they were suspected of containing dissolved air which might cause oxidation of some of the carotene or vitamin.

*Adsorbents*—The adsorbents were ultraporous solids, most of which were prepared in this laboratory. They were ground to pass a 200 mesh sieve and activated for 2 hours in a stream of carbon dioxide to remove air and moisture.

Alumina I was a gel made by adding a slight excess of  $\text{NH}_4\text{OH}$  to  $\text{AlCl}_3$  solution. The gel was allowed to dry slowly—over a period of 2 or 3 months—then washed free from chloride ion, dried again, and activated.

Alumina III was a commercial product of the Aluminum Company of America, made by slow crystallization from dilute  $\text{NaOH}$  solution, followed by heating to drive off most of the water of crystallization. The commercial product was carefully washed to remove any remaining traces of  $\text{NaOH}$ , but the wash water gave no alkaline test with litmus.

Alumina V was made by igniting the salt  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  for several hours at 200–300° until no more red-brown  $\text{NO}_2$  was given off. The resulting pure white solid passed a 200 mesh sieve without further grinding, and gave no test for nitrate ion with ferrous sulfate and sulfuric acid.

Alumina VI was made from aluminum ethoxide (7) which was broken up and exposed on a glass plate in a warm, moist room to secure hydrolysis. It was rolled and crushed with a glass rod once a week, and after 5 weeks, was removed. It was heated 3 hours in dry  $\text{CO}_2$  at 210° until no further condensation of vapor occurred in the exit tube of the activating flask. It was then ground and activated 2.5 hours in  $\text{CO}_2$ .

Alumina VII was alumina (7) deposited on pumice. The pumice was broken to 8 to 14 mesh pieces and mixed with an equal weight of aluminum ethoxide. The whole was then heated until the ethoxide melted (some of the latter was here lost as dense white fumes). After cooling, the melt was broken up and exposed to the air of a moist, warm room. After 2 weeks, it was removed and heated to 200° for 3 hours in dry CO<sub>2</sub>. It still appeared grayish in color and was therefore steamed 3.5 hours to accelerate hydrolysis, dried, and reheated at 200–230° for 2 hours.

Alumina VIII was a porous alumina made from aluminum butoxide (7) treated similarly to Alumina VI except that after exposure to the warm, moist air, it was steamed for 7 hours before being activated.

Patrick's silica gel of the commercial type (8), which had not been entirely freed from iron, was used.

Vitreous silica gel was made by the method of Holmes and Anderson (9). It was found very important to free acid-treated gels of every trace of acid. The gels were washed with water until apparently acid-free, then with 0.02 N NaOH, and again with water.

Norit carbon was purified by the hydrofluoric-hydrochloric acid treatment of Miller (10) to render it practically ash-free. It was activated in a silica crucible at 800–900° in an electric furnace as directed by Miller.

#### EXPERIMENTAL

##### *Methods*

*Vitamin A*—1 gm. of the activated adsorbent and 10 cc. of a solution of the vitamin were placed in bottles or tubes under an atmosphere of carbon dioxide. The bottles were shaken 10 minutes and allowed to stand overnight at room temperature to insure satisfactory settling. Blanks for each concentration were treated in all ways as in the regular runs except that porous solids were not present. In the numerous instances where duplicates were run they checked closely.

In order to recover the adsorbed vitamin the supernatant liquid was decanted and 10 cc. of chloroform added to the adsorbate while carbon dioxide was passed in. The tubes were then stoppered and, after shaking 10 minutes, allowed to stand overnight. In

the cases of adsorption from chloroform and ethylene dichloride a correction was made for the volume of liquid held mechanically by the adsorbent when the supernatant layer was poured off. This was not so satisfactory in the case of the petroleum ether solutions, however, owing to the extreme volatility of this solvent.

*Carotene*—Because of the high adsorption of carotene by some porous solids it was necessary in most cases to use 20 cc. of carotene solution with 1 gm. of adsorbent. To obtain some of the higher points on the curves 0.5 and 0.25 gm. samples of adsorbent were used with 20 cc. of solution, and the adsorption observed was multiplied by 2 or 4 respectively to obtain the specific adsorption.

The tubes were filled with carbon dioxide, the adsorbent rapidly weighed in, and carbon dioxide passed into the tubes again while the 20 cc. of solution were added. After closing the tubes with tin-foil-wrapped corks and shaking 10 minutes, the solutions were allowed to stand overnight to settle, although equilibrium was reached in much shorter time. The recovery was effected in the same way as in the case of vitamin A; that is, the supernatant liquid was poured off the adsorbent, and 20 cc. of chloroform were added, an atmosphere of carbon dioxide being maintained. The tubes were shaken 10 minutes and allowed to stand overnight before the concentration of the recovery solution was determined.

The amount of carotene present was determined colorimetrically with a dipping colorimeter. For the petroleum ether solutions the method of Willstätter and Stoll (11) with a standard 0.2 per cent solution of potassium dichromate was employed. For the solutions in other solvents it was found that the color of the carotene solution was shifted toward the red in chloroform and carbon disulfide especially, and would not match the dichromate standard, so it was necessary to make up known concentrations of carotene in the desired liquid. Ten readings were made and averaged in determining the concentration of each solution.

*Cholesterol*—With cholesterol adsorption, 20 cc. of solution and 2 gm. of activated adsorbent were used. The solutions in the various solvents contained 1, 2, 5, and 10 gm. per 100 cc. of total volume. The solutions were shaken 1 hour and then filtered through dry filter paper. The blanks were treated in the same way, except for omission of adsorbent. 10 cc. of each solution were evaporated on a water bath to constant weight, and the concentration of the

equilibrium solutions and of the blanks determined. The procedure followed in recovery was essentially the same as with the vitamin and carotene. The equilibrium solution was decanted and 20 cc. of chloroform added. The solutions were shaken 1 hour, filtered, and 10 cc. of the filtrate evaporated to constant weight to determine the equilibrium concentration.

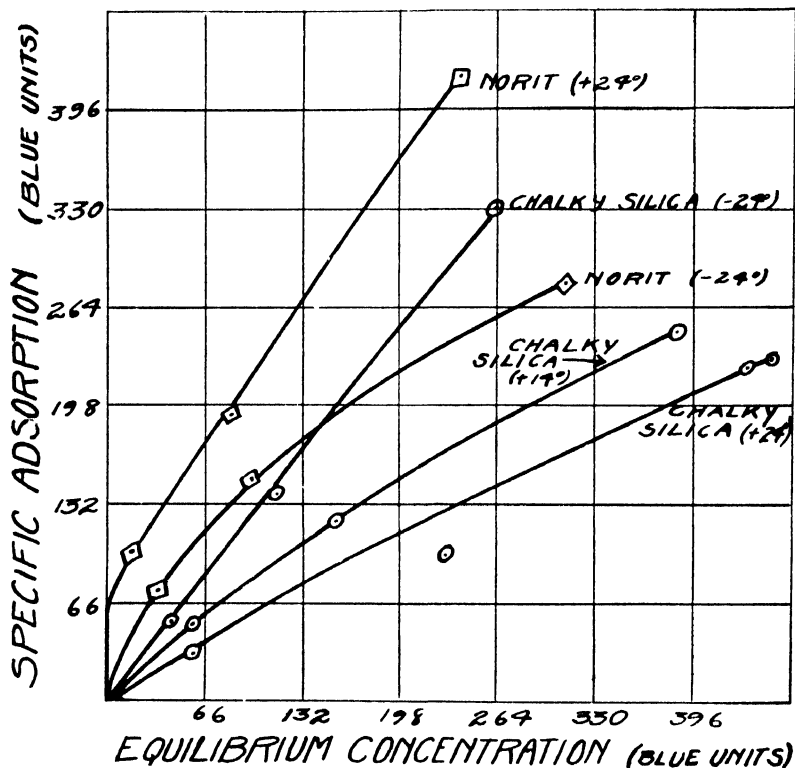


FIG. 1. Adsorption of vitamin A with non-fatty residue from chloroform. This graph indicates that in the case of the less chemically active silica, true adsorption (increasing with fall in temperature from  $+24^{\circ}$  to  $-24^{\circ}$ ) was the predominant influence with much less chemical reaction taking place, while with the more active norit carbon, chemical reaction (increasing with rise in temperature from  $-24^{\circ}$  to  $+24^{\circ}$ ) of some sort was decidedly the predominant influence, with true adsorption as a very minor phenomenon. The results represented in Fig. 1 were obtained by earlier work in which air was not rigorously excluded from the apparatus.

## Results

*Vitamin A*—Only one gel, Patrick's silica, excels Alumina III in ability to adsorb from petroleum ether. When these two porous

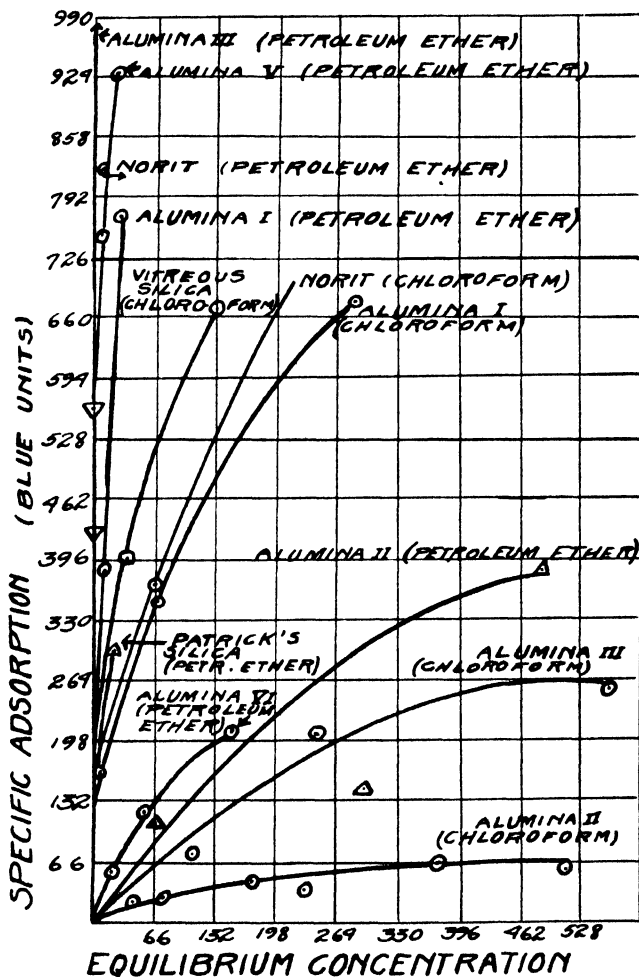


FIG. 2 Adsorption of crude vitamin A. It is evident that aluminas prepared in different ways vary widely in adsorbing power. Adsorption of crude vitamin A from petroleum ether by several porous solids is high but with carbon chemical reaction accounts for much of the removal from solvent. Alumina III is broadly effective.

solids are compared in their ability to liberate, however, Alumina III is far superior. Alumina V, which was made by igniting the nitrate, gave excellent adsorption and good recovery, but Aluminas VI and VIII, which were made by hydrolysis of aluminum ethoxide and aluminum butoxide respectively, each gave poor recovery,

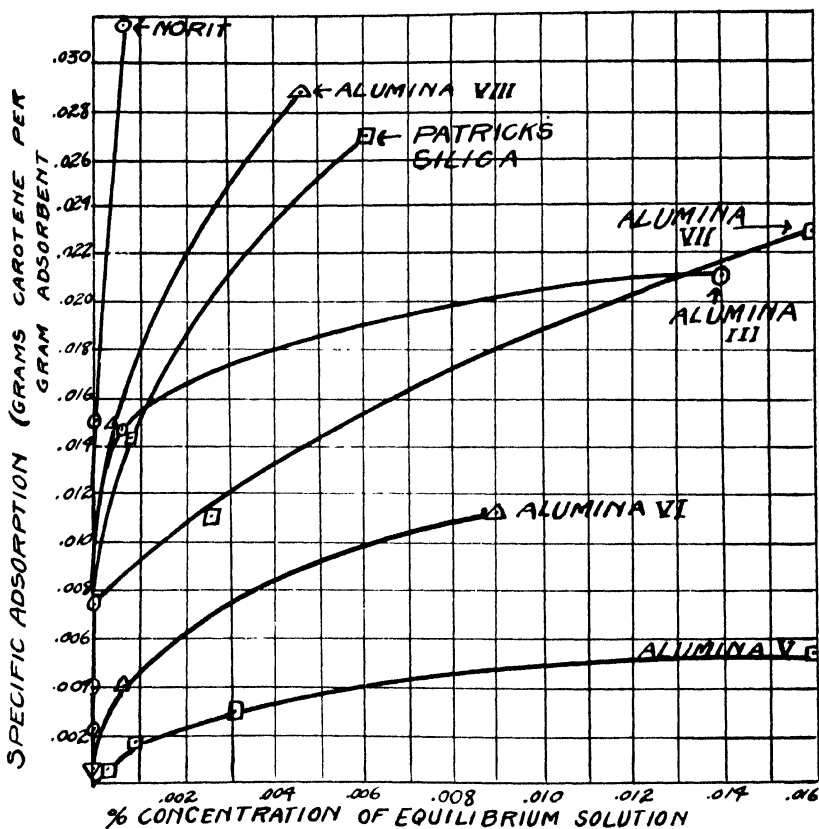


FIG. 3. Adsorption of carotene from petroleum ether

that from the ethoxide being higher in per cent than that from the butoxide. An analysis of these curves indicates that the use of Alumina III with petroleum ether as solvent for adsorption and chloroform for recovery should be the most effective combination (Fig. 2).

A recent article by Marcus (12) noted that contact with fine

powders destroyed the vitamin. The recoveries from several adsorbents make it evident that adsorption *per se* does not destroy it entirely, and in the case of Alumina III not to any marked extent.

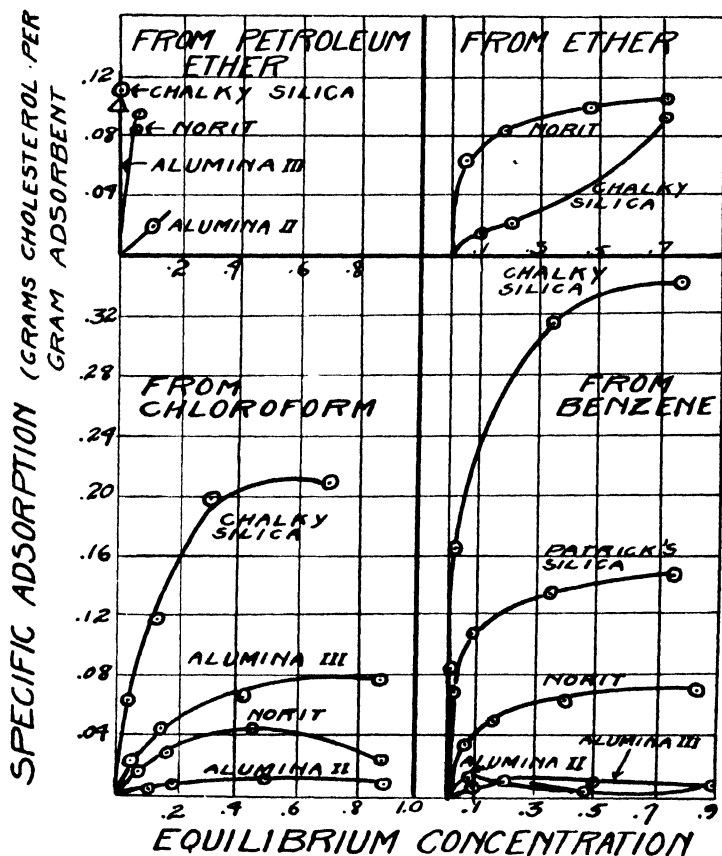


Fig. 4. Adsorption of cholesterol

**Carotene**—Some preliminary experiments were run with rather dilute solutions to determine which solvents were most, and which least effective for adsorption and recovery. It was found that adsorption from petroleum ether was excellent for nearly all adsorbents, thus paralleling the behavior of the vitamin, but from benzene, ethylene dichloride, and chloroform it was very poor

except in the case of norit, which showed some adsorption from benzene and even more from chloroform. More quantitative studies were made, therefore, with petroleum ether as solvent to adsorb from, and chloroform as liberating solvent, the same combination found most effective in the vitamin work.

In Fig. 3 it is shown that Alumina VIII is superior to Alumina III. The adsorption secured with Alumina V is very poor, in contrast with the high adsorption of vitamin A from petroleum ether by this adsorbent.

As in the case of vitamin A, the excellent adsorption shown by some gels was not followed by excellent recovery. The recovery secured from Alumina III was superior to that from Alumina VIII, as well as the recovery from any other gel studied.

*Cholesterol*—Cholesterol claimed our attention in these studies because of its presence in many sources of vitamin A, and because of the suggested chemical relationship between cholesterol and the vitamin.

The curves in Fig. 4 summarize the adsorption experiments made with cholesterol. The importance of the polarities of the components of the systems becomes apparent at once. Norit is non-polar, silica gel is polar and acidic, and alumina, polar and amphoteric. Silica gel adsorbs the slightly polar cholesterol quantitatively from petroleum ether, very highly from benzene, and in decreasing amounts down to ether, from which adsorption is low. The adsorption from benzene by chalky silica gel is much higher than by Patrick's gel; the adsorption by norit is decidedly lower, and shows a partial reversal of the order with silica gel. The polar ether and very non-polar benzene show the expected exchange of position clearly. The failure of petroleum ether solution to show a decided lowering of adsorption may be related to its slight solubility for cholesterol. The adsorption by alumina is lower, but follows the same order as that for silica. These results agree very well with the reversal of Traube's rule of adsorption as developed by Holmes and McKelvey (13).

Cholesterol in petroleum ether solution was found to be much like vitamin A in its adsorption behavior. For this reason the separation by adsorption of the vitamin from cholesterol and cholesterol-like matter in the non-saponifiable fractions of oils is a problem of great difficulty.

## SUMMARY

Several adsorption curves for vitamin A, carotene, and cholesterol are presented.

Attention is directed to a new type of porous alumina of low cost and great value for such adsorption work.

Adsorption from one solvent and recovery by another have been developed as an important research technique in vitamin work.

Contrary to some statements in the literature, a high percentage of vitamin A has been recovered from porous solids upon which it had been adsorbed.

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## AN EXTENSION AND A LIMITATION OF THE THIO-CYANATE METHOD FOR THE PREPARATION OF 2-THIOHYDANTOINS

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(Received for publication, October 26, 1932)

The method for the preparation of 2-thiohydantoins which Johnson and his coworkers (1, 2) have developed on the basis of the initial, distinctly misleading report of Komatsu (3) has proved quite generally useful. The present paper describes the application of the reaction to derivatives of  $\alpha$ ,  $\alpha'$ - and of  $\alpha$ ,  $\beta$ -diaminopropionic acid.

In the case of  $\alpha$ ,  $\alpha'$ -bis-acetaminopropionic acid (structure (I) in the accompanying formulas) the known azlactone (4) was presumably formed in the usual way, since the thiohydantoin (II) was readily formed under the usual conditions. When this was dissolved in dilute alkali and reprecipitated, it lost the 1-acetyl group and formed the thiohydantoin (III).

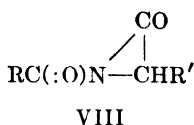
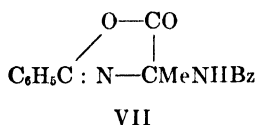
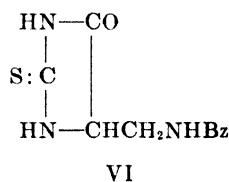
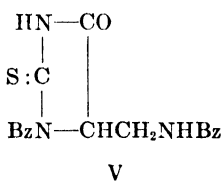
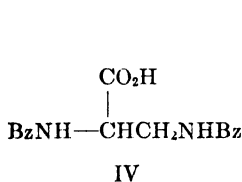
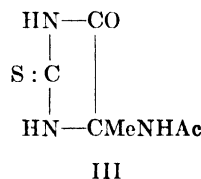
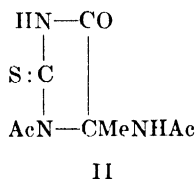
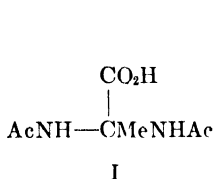
When the attempt was made to use the analogous  $\alpha$ ,  $\alpha'$ -bis-benzoylaminopropionic acid<sup>1</sup> for the analogous reaction, no thiohydantoin formation whatever could be demonstrated, and the original acid was recovered in good yield. The same was true when the pure azlactone (VII) of this acid was similarly used. This is, at first sight, a somewhat surprising result. The acid (I) and its benzoyl analogue are closely analogous substances and the reaction of thiohydantoin formation from  $\alpha$ -acylamino acids has been found to be of very general application. It will, however, be recalled that Johnson and Scott (2), who attempted to establish the mechanism of this interesting reaction, were puzzled by the fact that, while hippuric acid and its azlactone both readily yielded 1-benzoyl-2-thiohydantoin, both benzalhippuric acid and its

<sup>1</sup> To be described in a subsequent paper.

azlactone failed to give the corresponding reaction. The latter instance of failure has been confirmed by the writer, although Johnson and Scott ((2) p. 1140, foot-note) suggest that there may be conditions under which the reaction can occur.

The writer is inclined to accept as satisfactory the mechanism for the reaction given by Johnson and Scott (2). The instances of failure to react, however, seem to involve particularly highly substituted azlactones, or substitution with groups of considerable size. The absence of reaction in these cases can probably be best

*Formulas and Relationships of Compounds Discussed*



explained in accordance with the theories of Thorpe and Ingold (5), as due to a stabilization of the azlactone ring to such an extent that addition of  $\text{HNCS}$  no longer takes place. This is, to the writer, a much more attractive assumption than the idea (2) that certain azlactones may, for an unexplained reason, have the abnormal structure illustrated by (VIII), for which little if any evidence appears to exist.

There is, at least, certain definite experimental evidence that the azlactones which have been mentioned as failing to give thiohydantoins, are less capable of adding certain reagents than are

those that react successfully. The azlactone of  $\alpha$ ,  $\alpha'$ -bis-acetylaminopropionic acid is decomposed (6) on standing at room temperature with ethyl alcohol to give the unusual product  $\text{CH}_3\text{C}(\text{NH}_2)(\text{NHCOCH}_3)\text{CO}_2\text{H}$ . The azlactone (VII) of the corresponding benzoyl derivative can, however, be recrystallized from boiling alcohol without change. The azlactone of benzalhippuric acid is also unchanged on crystallization from alcohol (7).

It would be of decided interest in this connection to try the thiohydantoin reaction on the  $\alpha$ -acetylmino- $\alpha'$ -benzoylamino-propionic acid described by Bergmann and Grafe (6). As an intermediate case, this might or might not react under the usual conditions. In case of reaction, at least, it would be easy to tell which of the two different acyl groups is involved in azlactone formation, since only the one occupying position (1) in the resulting thiohydantoin would be readily removed by dilute alkali. The writer is not at present in a position to carry out this experiment.

In the case of  $\alpha$ ,  $\beta$ -dibenzoylamino-propionic acid (IV), the 1-benzoyl-2-thio-5-benzoylamino-methylhydantoin (V) and the corresponding 2-thio-5-benzoylamino-methylhydantoin (VI) were easily prepared.

#### EXPERIMENTAL

*1-Acetyl-2-Thio-5-Methyl-5-Acetylaminohydantoin (II)*—4 gm. of  $\alpha$ ,  $\alpha'$ -bis-acetylaminopropionic acid (4) and 2.5 gm. of ammonium thiocyanate were dissolved in 18 cc. of acetic anhydride and 2 cc. of acetic acid and the mixture was heated 30 minutes on a steam bath. The solution was then cooled and poured into 40 cc. of water. The crystalline product that separated weighed 2.3 gm., and on concentration of the mother liquors an additional gm. of cruder material was obtained. The substance crystallized from 10 parts of hot alcohol in white stubby prisms which melted definitely, when heated fairly rapidly, at  $230^\circ$ . The air-dried product, heated at  $100^\circ$  in a vacuum oven, lost 8.3 per cent in weight (calculated for  $1\text{H}_2\text{O}$ , 7.3 per cent); this dried material was analyzed.

*Analysis* (Kjeldahl)

$\text{C}_8\text{H}_{11}\text{O}_5\text{N}_3\text{S}$ . Calculated, N 18.32; found, N 18.2, 18.3, 18.2

*2-Thio-5-Methyl-5-Acetylaminohydantoin (III)*—0.5 gm. of the hydantoin (II) was dissolved, at room temperature, in 12 cc. of *N* sodium hydroxide, and after 30 minutes an equivalent amount of hydrochloric acid was added. The solution was evaporated at room temperature, and the dry residue extracted with 50 cc. of warm alcohol. The product formed prisms, m.p. 248°, with slight effervescence, after much preliminary darkening.

*Analysis* (Kjeldahl)

$C_8H_9O_2N_2S$ . Calculated, N 22.5; found, N 22.1, 22.0

*Attempt to Prepare Thiohydantoin from  $\alpha$ ,  $\alpha'$ -Bis-Benzoylamino-propionic Acid*—Application to this acid of the usual method resulted in the isolation of only one product, obtained in good yield, which was shown by mixed melting point and by analysis to be only the original acid. When the azlactone was prepared and treated similarly, the acid was again the only product obtained, and there was no indication of thiohydantoin formation.

*1-Benzoyl-2-Thio-5-Benzoylaminomethylhydantoin (V)*—1 gm. of the acid (IV) gave, under the usual conditions of reaction, slightly more than 1 gm. of the hydantoin (V), which was very difficultly soluble in water, and only rather slightly soluble in hot alcohol. It was best crystallized from 10 parts of hot acetic acid. The sulfur-yellow, crystalline powder melted at 211–212° with effervescence and darkening.

*Analysis* (Kjeldahl)

$C_{18}H_{16}O_4N_2S$ . Calculated, N 11.90; found, N 11.78, 11.66

*2-Thio-5-Benzoylaminomethylhydantoin (VI)*—0.5 gm. of the hydantoin (V) was dissolved in 0.5 *N* sodium hydroxide, and after 5 minutes at room temperature the solution was acidified with hydrochloric acid. The white crystalline precipitate was washed with water and with benzene (to remove benzoic acid). After crystallization from hot glacial acetic acid, in which it was difficultly soluble, it melted at 255° with decomposition.

*Analysis* (Kjeldahl)

$C_{11}H_{11}O_2N_2S$ . Calculated, N 16.86; found, N 16.4

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# THE ELIMINATION OF BROMIDES FROM THE BLOOD STREAM\*

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Although much attention has been devoted to questions relating to the distribution of bromides in the animal body, no attempt appears to have been made to examine the behavior and fate of bromides, present in concentrations comparable with those observed in clinical conditions, over long periods of time. Such a study is here reported.

## EXPERIMENTAL

Two dogs, weighing 15 and 20 kilos respectively, were employed for the experiments.

Bromide in blood and urine was determined by the method of Behr, Palmer, and Clarke (1); total halide was estimated in aliquot portions of the alkaline ash solutions by the Harvey modification of the Volhard procedure (2). Carbon dioxide was estimated in serum (collected under oil) by the procedure of Van Slyke and Neill (3), with the closed manometer apparatus. Total fixed base determinations were made by the procedure of Stadie and Ross (4). Urine was collected without catheterization.

The salt-poor diet upon which the dogs were kept consisted of ground dog biscuit with the addition of a 1 per cent solution of extract of beef and 2.5 cc. of cod liver oil daily for each dog. Tap water was allowed *ad libitum*. On this diet the dogs received

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† George Blumenthal, Jr., Fellow in Columbia University, 1929-31. The data in this paper are taken from a thesis submitted by John W. Palmer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

about 10 mm of chloride daily. In determining the degree of replacement of chloride in cells and plasma, a 1.1 per cent solution of potassium oxalate was found to give a minimum of both hemolysis and crenation with the blood of both dogs employed. This is more dilute than that adopted by Hooper, Smith, Belt, and

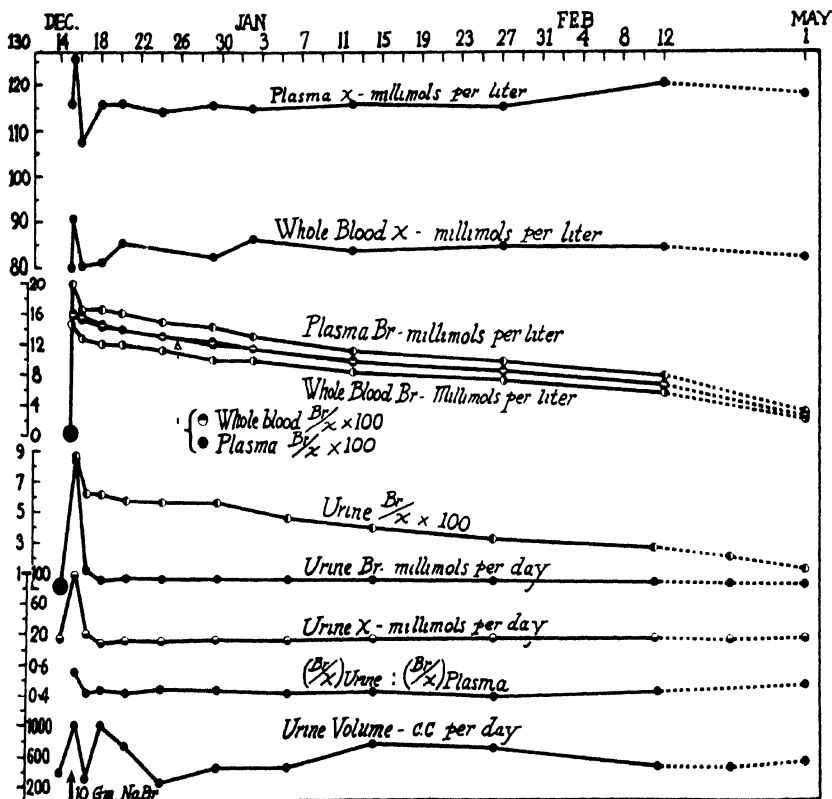


FIG 1 Intravenous NaBr

Whipple (5). Blood samples were collected under oil and immediately mixed with the above isotonic oxalate solution, also under oil.

In the first experiment (Fig. 1), 10 gm. (97.2 mm) of sodium bromide in 100 cc. of solution were injected into the right femoral vein of a 15 kilo dog, the injection being complete in about 10

minutes.<sup>1</sup> The halide content of the blood and urine was studied over a period of 4½ months.

The extreme slowness of the excretion of bromide on a low chloride diet is apparent: 30 minutes after the injection was complete, the blood contained nearly 15 mm of bromide per liter; 4½ months later there were still 2 mm per liter. During that time

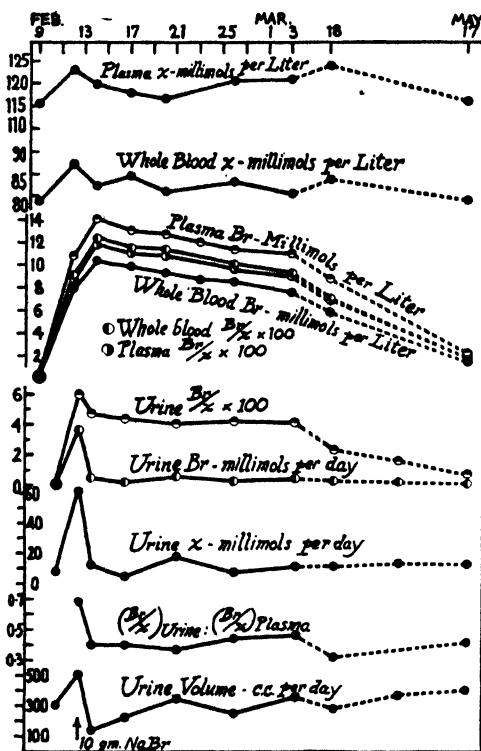


FIG. 2. Peroral NaBr

the molecular percentage of bromide in the total halide dropped from 15.90 to 2.43. This percentage is the same in plasma and whole blood, any differences being within the limits of error.

During the same period the excretion of bromide fell from 8.35 to 0.135 mm per day, and the molecular percentage of bromide in the

<sup>1</sup> No ill effects were ever observed following the slow injection of highly hypertonic solutions.

halide of the urine dropped from 8.63 to 1.1. During the 1st day after the dose of bromide the increase in excretion of chloride is molecularly equivalent to 80 per cent of the bromide given, while the bromide excretion is 8.6 per cent.

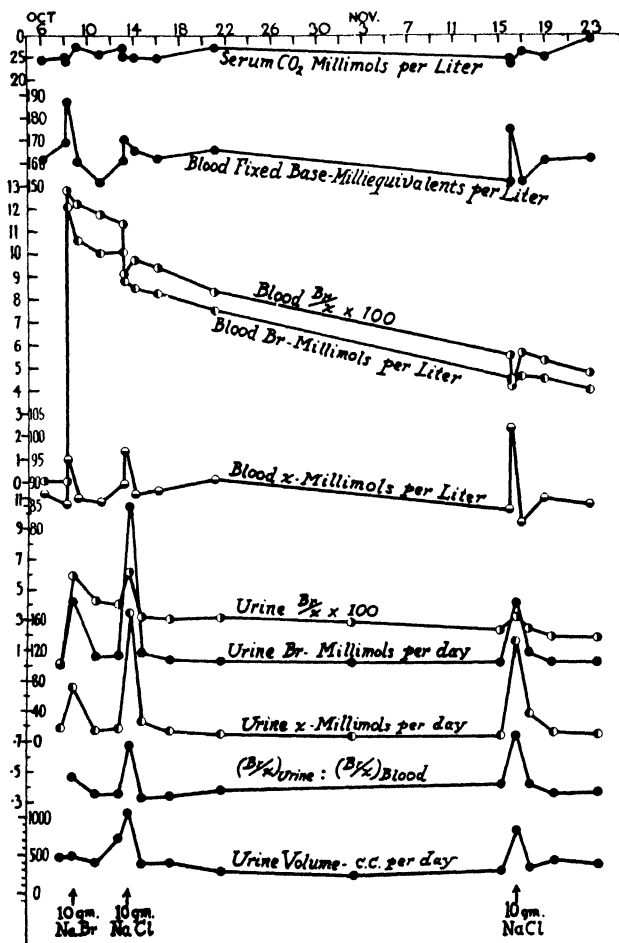


FIG. 3. Intravenous NaBr and NaCl

The constancy of the ratio  $(\text{Br}/\text{X})_{\text{urine}} : (\text{Br}/\text{X})_{\text{plasma}}$  which was except for the 1st day, around 0.4 is striking.<sup>2</sup> During the 1st

<sup>2</sup> Throughout this paper, X represents total halide.

day, while the large amount of chloride was being excreted, this ratio was 0.55, but throughout the rest of the experiment it varied only between 0.38 and 0.45.

The second experiment (Fig. 2) differed from the first only in that the bromide was given by mouth to a 20 kilo dog. The food containing the salt was eaten in about 3 hours. The highest bromide level in the blood was reached during the period  $\frac{1}{2}$  to 24 hours after ingestion. In this experiment the same constancy of  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{plasma}}$  was observed after the 1st day: the variations were from 0.32 to 0.46, with most of the figures between 0.37 and 0.44.

The third experiment (Fig. 3) was devised to study the effect of comparatively large doses of sodium chloride while the animal (20 kilo) still contained fairly large amounts of bromide. All salt injections in this and the following experiment were made slowly into the right jugular vein, with 10 gm. of the salt dissolved in 100 cc. of solution.

The carbon dioxide content was followed as a measure of the effect of the halide doses upon the other anions normally present in blood, and the fixed base was followed for the effect on the cations. For all analyses except  $\text{CO}_2$  the blood was collected over dry ammonium oxalate. The  $\text{CO}_2$  content of serum was studied rather than that of whole blood, as the analytical procedure is simpler; this is justified by the observation of Atchley and Benedict (6) that any  $\text{CO}_2$  change in whole blood is paralleled by a similar change in the serum. Small decreases in  $\text{CO}_2$  were found about a half hour after the injections, but these decreases were smaller than the usual variations from day to day.

The fixed base of the blood rose, as expected, with the injection of the salt solution, and rapidly returned to the original value. No satisfactory explanation for the abnormally high rise with the bromide injection, nor for the drop below normal after this, can be offered at present.

The established fact that chloride can displace bromide is illustrated by the greatly increased bromide excretion immediately following the chloride dose. This effect was transitory, and subsided as soon as the excess chloride was eliminated. The constancy of the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  with constant chloride intake was again observed, and high ratios were found after the injections of both chloride and bromide.

Since these injections always produced a marked diuresis, it was desirable to know whether the same effect on the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  could be brought about by an increase in urine volume caused by other means. In the fourth experiment (Fig. 4), 100 cc. of a solution containing 25 gm. of urea, which

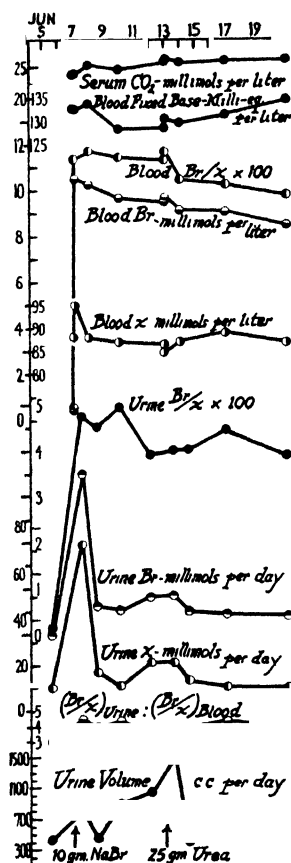


FIG. 4. Intravenous NaBr and urea

causes a diuresis in much the same manner as sodium chloride, were injected into the 20 kilo dog after a corresponding interval. That the total fixed base in the blood is much lower than in the previous experiment may be attributed to the fact that the dog

had been on the low salt diet for about 7 months when this experiment was begun. That the first blood specimen taken after injection of bromide contains less fixed base than the second is due to the inadvertent injection of a considerable part of the bromide solution into the tissue surrounding the vein. The apparent diuresis immediately preceding the urea injection was due to the spilling of drinking water by the dog during periods of restlessness.

Injection of urea was without effect on the amount of either chloride or bromide excreted, and the ratio  $(\text{Br}/\text{X})_{\text{urine}}:(\text{Br}/\text{X})_{\text{blood}}$  was not changed.

#### DISCUSSION

The first two experiments clearly demonstrate that after equilibrium is established, the cell walls do not differentiate between bromide and chloride. This is in agreement with the findings of Bönniger (7) and of Woodhouse and Pickworth (8).

Hastings and van Dyke (9) have shown that *in vitro* bromide passes readily between cells and serum, but the ratio  $(\text{Br})_{\text{cells}}:(\text{Br})_{\text{serum}}$  is always a little higher than the ratio  $(\text{Cl})_{\text{cells}}:(\text{Cl})_{\text{serum}}$ . When, however, dogs were given large doses of bromide by mouth (10), the corresponding bromide ratio was at first much higher than the chloride ratio, reaching a maximum about a half hour after the dose; it then fell until the *in vitro* ratios were reached. Hastings and his collaborators (10, 11) therefore concluded that the red blood cells have an especial attraction for bromide ion *in vivo*. However, the work of these investigators covers a relatively short period of time after heavy dosage with bromide, while the present data relate to equilibrium values at much lower concentrations.

The present data show that the percentage of bromide in the urinary halides is lower than that in the blood halides. The kidney, therefore, preferentially excretes chloride. Hastings, Harkins, and Liu (11) in a few instances obtained indications of this, but the time of their experiments was too short to allow them to observe the effect to its full extent.

No record, nor even suggestion, of the constancy of the relation  $(\text{Br}/\text{X})_{\text{urine}}:(\text{Br}/\text{X})_{\text{blood}}$  on constant chloride intake appears in the literature. With an intake of about 0.6 mm of sodium chloride

per day, per kilo of body weight, this ratio had a value of about 0.4. Not only does the kidney excrete a smaller proportion of the bromide than of the chloride, but the magnitude of this proportion appears to depend on the level of chloride intake.

When the excretion of total halide is increased by administration of either bromide or chloride, the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  also rises. The bromide ion moves slightly more rapidly than the chloride ion (12); if, therefore, the secretion of urine were a simple osmotic process, the above ratio should be greater than unity. On the other hand, there is good evidence (13) for the view that the secretion of the urine consists of filtration of the plasma in the glomeruli of the kidneys, followed by concentration in the tubules, with some reabsorption of dissolved halide (14). On this basis the bromide should be more rapidly reabsorbed than the chloride. The preferential excretion of the chloride thus appears to depend upon the extent of reabsorption in the tubules. Theoretically, by producing a high enough halide excretion, a point could be reached where the ratio  $\text{Br}:X$  would be nearly the same in urine and blood. Frey (15) has recorded values which indicate such a condition in rabbits under the influence of phlorhizin and at the height of diuresis induced by water, sodium sulfate, and sodium nitrate. This point was not reached in the present work, the highest value of the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  being 0.73, obtained in a 20 kilo dog after injection of 171 mm of sodium chloride. This was at a low level (4.38 mols per cent) of bromide in the blood; at a higher level (9.17 mols per cent) the ratio was nearly the same (0.67) after injection of the same quantity of sodium chloride. The ratio thus depends upon the amount of halide being excreted, and is independent of the blood bromide level. With low (10 mm per day) salt intake, the ratio remains consistently around 0.4.

We find no relation to exist between urinary volume and bromide excretion. Following the chloride injections (Fig. 3) the excretion of bromide and total halide, the urinary volume, and the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  all rose; after the urea injection (Fig. 4) there was an increase in urinary volume comparable to that caused by the chloride injection, but the other figures showed no corresponding rise. After administration of chloride, the changes observed (Fig. 3) in the bromide excretion and in the ratio  $(\text{Br}/X)_{\text{urine}}:(\text{Br}/X)_{\text{blood}}$  are therefore the result of a rise in

halide excretion and not of changes in urinary volume *per se*. These results serve to explain the clinical observation (16) that bromide dermatitis responds to the administration of chlorides but not of other diuretics. No explanation can be offered for the lack of agreement with the observations of Frey (15).

In the treatment of bromism the principal concern is to supply as much chloride as possible, since chloride not only increases the halide excretion but increases the fraction of bromide in it. Through the courtesy of Dr. J. G. Hopkins it was possible to study a few patients with bromide dermatitis. It was not feasible to make as complete a study with patients as with experimental animals, but a few interesting figures were obtained. One patient showed on admission a blood bromide value of 17.63 mm per liter, representing 21.4 mols per cent of the total halides (82.5

TABLE I  
*Halide in Normal Human Urine*

Br	X	100 Br/X
<i>mm per l.</i>	<i>mm per l.</i>	
0.046	16.21	0.28
0.059	14.48	0.41
0.066	18.44	0.36
0.102	19.22	0.53
0.024	22.38	0.11

mm per liter). After a week of treatment (consisting of oral administration of 5 gm. of NaCl daily in addition to the salt of the diet) the lesions all showed a decided improvement. The blood then contained 80.4 mm per liter of total halides, comprising 11.17 mm of bromide or 13.9 per cent of the halide. At this point a 24 hour urine specimen showed 167 mm of halide containing 10.3 per cent (17.2 mm) of bromide. The value of the ratio  $(\text{Br}/\text{X})_{\text{urine}} : (\text{Br}/\text{X})_{\text{blood}}$  was thus 0.74.

Another such patient was found to have a blood bromide of 19.49 mm or 22.85 per cent of the 85.3 mm of total halide per liter. After 10 days without especial treatment other than high salt diet, the blood bromide had fallen to 10.58 mm per liter or 12.78 per cent of the 82.7 mm of total halide per liter. Corresponding urine specimens showed respectively 16.12 and 6.79 mm of bromide

per liter, 105.3 and 71.0 mm of total halide per liter, or 15.31 and 9.56 per cent of the halide as bromide. The ratio  $(\text{Br}/X)_{\text{urine}}$ :  $(\text{Br}/X)_{\text{blood}}$  had thus risen from 0.67 to 0.74 during the interval.

For comparison, there is included Table I, showing analytical results secured from the urine of five normal individuals giving no history of bromide medication.

#### SUMMARY

1. Bromide replaces chloride to an equal extent in blood cells and plasma.

2. The kidneys excrete chloride preferentially over bromide.

3. The ratio of the fraction of bromide in total halide in the blood to that in the urine is constant during constant chloride intake. When the chloride intake is raised, this ratio also rises. With a chloride intake of about 0.5 mm per day per kilo of body weight, this ratio has a value of about 0.4; with 18-fold chloride intake, the ratio rises to about 0.7.

4. Bromide excretion is not affected by changes in urinary volume produced by dosage with urea.

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## A MICRO CONDUCTANCE CELL\*

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Bayliss and Walker (1930) in an investigation of glomerular fluid described a conductivity cell which permitted determinations on a few tenths of a c.mm. of material. The micro cell of Remesow (1929) required 50 c.mm. of fluid. The design of the cell described here suggested itself during work involving the sealing of bridges of small bore Pyrex capillaries between larger tubes. The design is believed to possess certain advantages over that of Bayliss and Walker, although the latter performed its task satisfactorily. The principle is to join the female elements (*A*, Fig. 1) of two interchangeable ground glass joints by a short length of capillary tubing (*B*) of 6 to 7 mm. outside diameter. The male elements (*C*) bear hollow glass seals (*D*) carrying the platinum electrodes (*E*) which make contact with the mercury columns (*F*). The large bore bridge (*G*) insures pressure equilibrium, avoiding the possibility that the droplet in the capillary be pushed to one end, and also serves as a convenient handle. In use the two male elements bearing the electrodes are removed and the middle segment, bearing the capillary cell, is cleaned and dried. Moist filter paper is placed on the inner walls of the bulb on either side of the capillary, to insure saturation with water vapor. The micro pipette containing the liquid under investigation is touched to an end of the capillary and its contents expelled. No difficulty with bubbles, sticking, evaporation, etc., is experienced in this process, as the liquid immediately jumps across the capillary segment. The pipette is withdrawn and the male elements bearing the electrodes inserted until they are firmly seated. This auto-

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matically insures that the ends of the platinum wires will be fixed at a constant distance from each other within the column of liquid in the capillary. The capillary bore is flared at each end, facilitating the insertion of the electrodes. Repeated determinations have shown that variations due to inconstancy in seating of the male elements are not more than 0.2 per cent of a 1.3 mm. inter-electrode distance. Copper wires dipping into the mercury columns make the leads from the cell to the Wheatstone bridge.

This design possesses the following advantages.

1. The only materials with which the liquid under investigation comes into contact are glass and the electrodes.

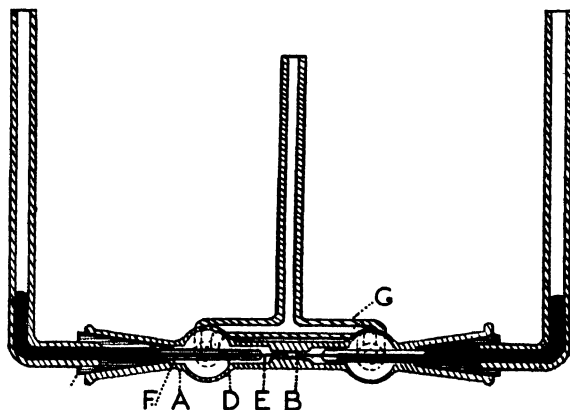


FIG. 1. Micro conductance cell. *A* represents the female elements; *B*, capillary tubing; *C*, male elements; *D*, glass seals; *E*, platinum electrodes; *F*, mercury columns; *G*, large bore bridge.  $\times \frac{1}{2}$ .

2. Transfer of the liquid from the containing pipette into the capillary cell is easy and all possibility of evaporation is avoided. This has been repeatedly checked by showing that no change in resistance occurs on standing in the cell for over an hour. Further, if a great excess of known KCl solution is used, so that the space on each side as well as the capillary cell itself is filled, the resistance is the same as when the cell is filled with 0.2 or 0.3 c.mm. from a micro pipette.

3. The smoothing of two vulcanite blocks so that they will face uniformly to give a constant interelectrode distance, as in Bayliss and Walker's cell, is not an impossible achievement, as

they have shown. Nevertheless, it demands a high degree of workmanship which in the present design is delegated to the makers of the ground glass joints.

4. Cleaning the cell and replatinizing the electrodes are simple procedures.

5. The cell can conveniently and safely be immersed in a constant temperature bath.

6. The resistance even with very small amounts of biological fluid is relatively high. If we take 0.1 N KCl as corresponding to about an average concentration, the resistance can be kept around 5000 ohms. Thus, with a cell designed to take 0.25 c.mm. of material, a tube of 0.40 mm. diameter and about 2 mm. length of uniform bore is taken. With an interelectrode distance of about 1.3 mm. the resistance with 0.1 N KCl is about 5000 ohms. If a larger amount of material is available, a capillary of correspondingly greater bore and length can be chosen so as to keep the resistance around 5000 ohms. While Bayliss and Walker do not give their cell constant, a consideration of their cell dimensions shows that the resistance with 0.1 N KCl must have been considerably less than 5000 ohms. The matter of resistance becomes of importance because of the small electrode surface in both of these types of cells. The greater the current flow per unit of electrode area the greater the polarization with consequent lessening of the accuracy of the measurement. Kohlrausch and Holborn (1916) obtained satisfactory minima with the telephone with resistances as low as  $15/n$ , where  $n$  is the area of one electrode in sq.cm. The area of Bayliss and Walker's electrode was the cross-sectional area of a wire of 0.3 mm. diameter, or about  $7 \times 10^{-4}$  sq.cm., demanding a resistance of 20,000 ohms to satisfy this requirement. In the design described here, with a 0.4 mm. capillary a 0.28 mm. platinum wire is used as the electrode. Since, however, the wire extends into the column of liquid, the electrode area is several times that of the wire's cross-sectional area. If we assume it to be 4 times (a low estimate), we see that, since a column of 0.1 N KCl of 0.4 mm. diameter and 1.3 mm. length has a resistance of about 8500 ohms, the above condition is satisfied in this cell. This still holds even though the resistance is lowered to somewhere around 5000 ohms by the conductance around the sides of the wire in the droplet. The fact that Bayliss

and Walker could read their minima to about 0.5 per cent shows that their excess polarization above that present when Kohlrausch and Holborn's condition is fulfilled still permits a reasonable accuracy.

With a fixed bridge ratio of 1:1 and a 10,000 ohm dial resistance box, with a 1000 cycle microphone hummer as the source of current, the end-point is not very satisfactory. Consecutive readings will vary by about 1 per cent, the average of several readings having a probable error of 0.3 to 0.5 per cent. With a Kohlrausch slide wire and a high frequency (1000 cycle) generator the minimum is much sharper and consecutive readings do not vary by more than 0.2 per cent, with a probable error of an average of several readings of 0.1 per cent. The capacity of the cell is very low; however, a 500 micro-microfarad variable air condenser across the resistance box improves the end-point, only a small fraction of

TABLE I  
*Results with Known Solutions of Various Concentrations*

Concentration of KCl solution	Observed resistance (25°)	Cell constant
<i>N</i>	<i>ohms</i>	
0.2	2584	0.01563
0.1	4954	0.01566
0.05	9560	0.01572

this capacity being needed. If the insertion of the electrodes into the capillary cell is done with reasonable care they can be used repeatedly without replatinizing.

Some results on known KCl solutions are given in Table I. The specific resistance of 0.2 N KCl at 25° is taken as 40.4, of 0.1 N as 77.8, and of 0.05 N as 150.3 ohms. These measurements were not at constant temperatures but the temperature was recorded and the resistance calculated as at 25° by the equation  $R_{25} = R_t / (1 - 0.022(t - 25))$ . Still closer agreement could probably be obtained at constant temperature. The measurements shown in Table I were made with the microphone hummer and fixed bridge ratio.

It is seen that the cell constant changes but little through a range from 0.2 N to 0.05 N, which covers most biological fluids. The data in Table I were obtained with a cell which permits

measurement with 0.2 c.mm. of material. It seems unnecessary to report here the variety of measurements that have been made on known salt solutions.

It is hoped that this cell will fill a need in biological work. Since the question of the identity of the molecular concentrations of glomerular fluid and of plasma seems to be settled in the affirmative, I have not made any measurements on glomerular fluid but have designed the smallest cell so that it can be used with easily available quantities of this fluid, with the idea that such volumes represent somewhere near the lower limit for practical handling. In comparison with 0.2 c.mm. enormous quantities of such fluids as sweat, cell sap, aqueous humor, edema fluid, tears, etc., are easily available.

This cell, with capillary cell dimensions according to any specifications given, will be fabricated by and can be purchased from Mr. W. J. D. Walker, Washington University, St. Louis.

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## THE USE OF METAL CAGES IN THE STUDY OF NUTRITIONAL ANEMIA\*

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(Received for publication, September 13, 1932)

As our past studies on nutritional anemia (5, 7) have been conducted on rats in glass cages, the recent report of Skinner, Steenbock, and Peterson (6) that metal cages are superior to glass cages for such studies has been of extreme interest to us. Only a few other workers have reported the use of both glass and metal cages for the study of nutritional anemia. Although Keil and Nelson (3), and Beard and Myers (1) do not agree concerning the value of iron alone as a stimulant for hemoglobin regeneration, both groups of workers have obtained consistent results whether galvanized iron or glass cages have been employed. Nevens and Shaw (4) have found that sufficient metals may be obtained from common iron wire cages to cause a regeneration of hemoglobin in rats on an exclusive milk diet without the addition of metallic supplements, whereas animals kept in new galvanized iron cages or in specially designed glass cages develop a severe anemia. The purpose of this paper is to report a study, completed just as the article of Skinner, Steenbock, and Peterson appeared, to determine whether metal cages could be used as satisfactorily as glass cages for investigations on nutritional anemia.

\* The experimental data in this paper are taken from the thesis submitted by Gladys B. Geraghty to the Graduate School of the University of Colorado, August, 1932, in partial fulfilment of the requirements for the degree of Master of Science.

A preliminary report of this paper was made before the Division of Biological Chemistry at the meeting of the American Chemical Society at Denver, August, 1932.

## EXPERIMENTAL

In general the method of Elvehjem and Kemmerer (2) for the production of nutritional anemia in albino rats was followed. Rats, 13 to 19 days old, were placed with their mothers on an exclusive whole cow's milk diet. Once a day the mothers were removed to another cage and were fed the diet of supplemented grains used for our stock colony; before being returned to their litter, they were carefully brushed. On about the 21st day the young rats were weaned and placed in glass cages. At 25 to 32 days of age, each litter was equally distributed, as far as possible, into individual cages of three types. Besides the glass cages used in our previous anemia studies (7), new galvanized iron wire cages and old galvanized iron wire cages were studied. The latter had been used for several years and showed some evidence of rust in every instance. All of the wire cages, both old and new, had false screen bottoms of  $\frac{1}{2}$  inch mesh. An exclusive diet of whole cow's milk was fed until the animals developed a severe anemia. The milk was collected into glass containers which had been carefully washed, as described in another publication (5), to eliminate any possibility of contamination. The animals were weighed weekly, and hemoglobin determinations were made bi-weekly with the Newcomer hemoglobinometer. Blood was obtained from the tail; excessive bleeding was prevented by touching the point of incision with a hot glass plate. When the hemoglobin had fallen to a level of about 4.0 gm. per 100 cc. of blood, 0.5 mg. of iron as ferric chloride was added each morning to a small amount of whole milk and, when this was consumed, additional milk was fed *ad libitum*. The ferric chloride, prepared by a method similar to that previously described (7), was found by spectroscopic examination<sup>1</sup> to contain no trace of copper.

*Results*

All of the rats, whether in glass cages (ten animals), old metal cages (nine animals), or new metal cages (nine animals), promptly developed nutritional anemia. A comparison of the rates at which this occurred in the different cages may be made from the data obtained. As in some cases iron was added as a supplement

<sup>1</sup> We are indebted to Professor H. B. Van Valkenburgh of the Department of Chemistry for the spectroscopic analysis of the ferric chloride.

to the milk diet before the hemoglobin level had fallen to 4 gm. per 100 cc. of blood, the results with these animals have not been included in this study of the rate of development of anemia. Of the rats whose hemoglobin was allowed to fall below 4 gm. per 100 cc. of blood, the average time required for the hemoglobin to reach

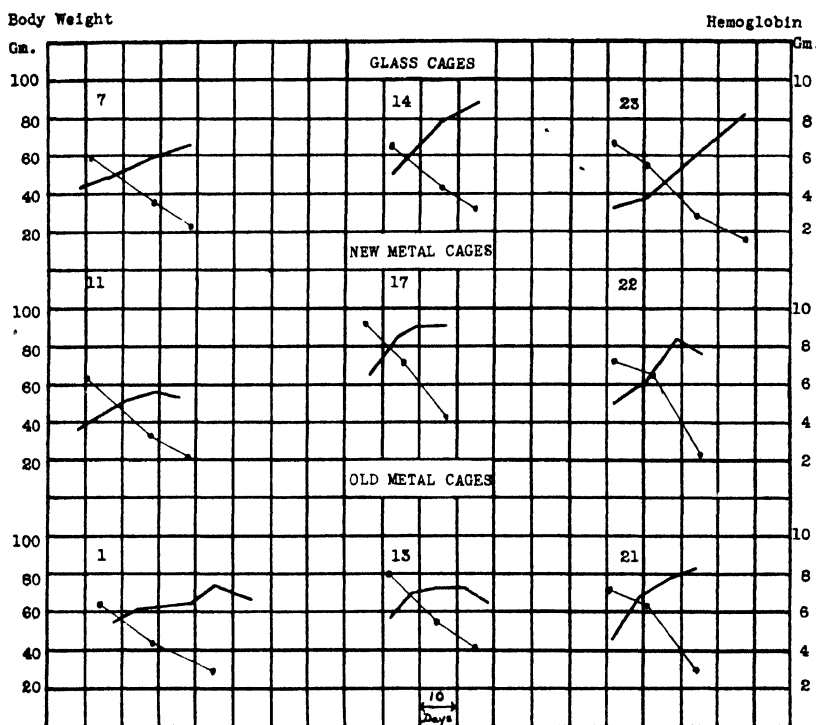


CHART I. Comparative rate of development of nutritional anemia in albino rats kept in glass cages, new metal cages, and old metal cages, respectively, and fed a diet of whole milk. Hemoglobin curves are represented by light lines with dots to indicate individual determinations; growth curves, by heavy solid lines. The identification number of each animal is given above its corresponding set of curves.

this level was 51 days for six animals in glass cages, 49 days for seven animals in old metal cages, and 46 days for seven animals in new metal cages. The differences here observed are hardly significant, particularly in view of the fact that the number of

Body Weight

Hemoglobin

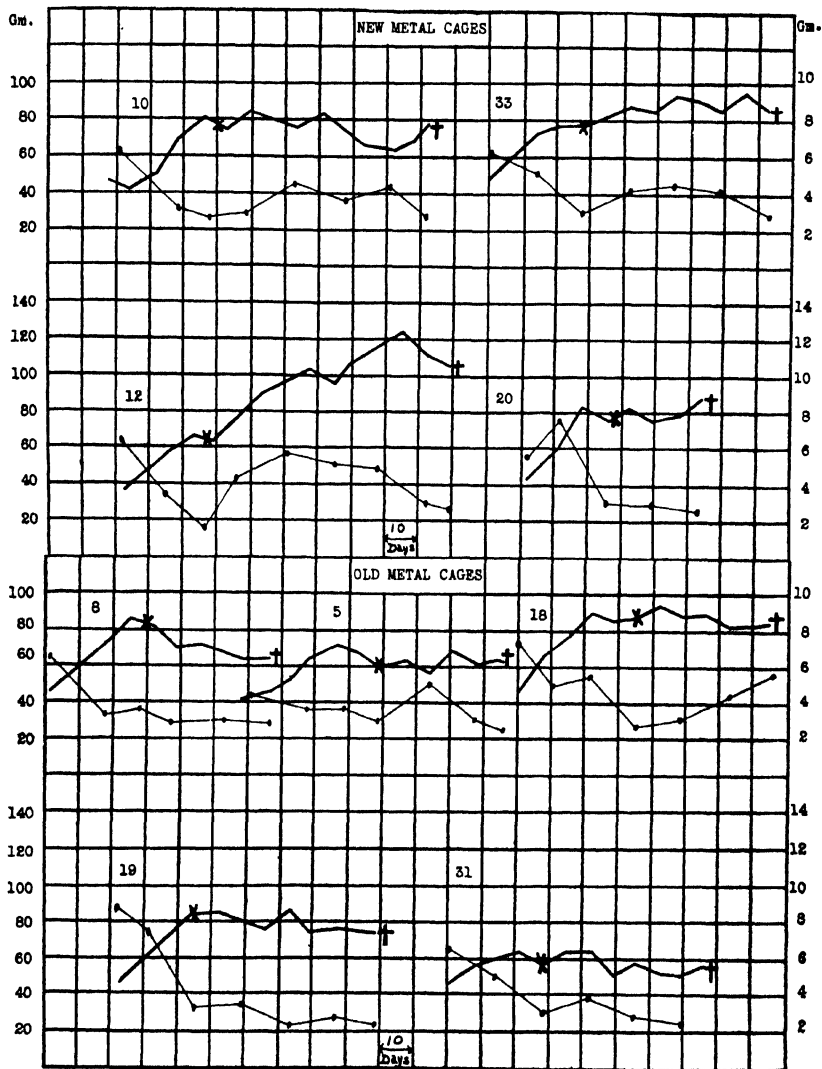


CHART II. Failure of iron when fed as a supplement to a whole milk diet to cause regeneration of hemoglobin (light lines with dots to indicate individual determinations) in albino rats kept in new and old metal cages, respectively. The cross on the growth curves (heavy solid lines) shows the time at which the addition of iron (0.5 mg. daily) was begun. The dagger indicates death. The identification number of each animal is given above its corresponding set of curves.

animals in each group was small and individual physiological variation would be expected to have some effect on these average figures. Chart I gives characteristic growth and hemoglobin curves for three typical animals from each kind of cage used.

Chart II gives the growth and hemoglobin curves of animals kept in old and in new metal cages, respectively. It may be observed that, when iron alone is added as a supplement to an exclusive milk diet, regeneration of hemoglobin does not occur in anemic rats kept either in new or in old metal cages. These results are quite in accord with those obtained with glass cages both previously (7) and in this study. The latter data are omitted from this paper to conserve space.

#### DISCUSSION

Skinner, Steenbock, and Peterson (6) ascribed their failure to produce anemia in young rats kept in glass cages and fed a milk-iron diet to increased coprophagy resulting from adhesion of excreta to the tubes forming the bottoms of their glass cages. By inference, the impression might be gained that the results reported by us during the past 2 years on the effect of metals in nutritional anemia have been vitiated by the fact that all of our experiments, with the exception of those herein described, have been conducted on rats housed in glass cages. The data of earlier papers (5, 7) are offered as proof that coprophagy as the result of using glass cages could not have been a significant factor in our studies. We found consistently that iron, given alone or with certain inert metals as a supplement to milk, did not cure or prevent nutritional anemia in young rats, thus establishing the suitability of our glass cage for anemia studies. The failure of our cumulative data to support the observation of Skinner, Steenbock, and Peterson (6) that glass cages are less suitable than metal cages for studies on nutritional anemia may well be due to the difference in the type of glass cage used.

#### SUMMARY

1. Nutritional anemia was produced by feeding whole milk to rats housed in glass cages, old galvanized iron wire cages, and new galvanized iron wire cages, respectively, at essentially the same rate.

2. Purified ferric chloride when added to an exclusive milk diet did not cause regeneration of hemoglobin in anemic rats kept either in old or in new metal cages. These results are completely in accord with our repeated observations on rats in glass cages.

3. Galvanized iron wire cages appear to be as suitable as our glass cage for nutritional anemia studies.

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## BLOOD VOLUME STUDIES IN COBALT POLYCYTHEMIA\*

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Our earlier work on polycythemia demonstrated that cobalt when given with copper to rats on a milk-iron diet produces a polycythemia characterized by a marked increase in the hemoglobin, erythrocyte, and cell volume values. The question then logically arose as to whether cobalt produces a *relative* polycythemia by causing a decrease in blood plasma volume, an *apparent* polycythemia by inducing a redistribution of inactive cells, or a *true* polycythemia by bringing about an actual addition of erythrocytes to the circulation. Obviously, a knowledge of plasma, cell, and total blood volumes is essential to answer this question, and, accordingly, these determinations were made on our cobalt-fed polycythemic rats.

### EXPERIMENTAL

The vital red method of Keith, Rowntree, and Geraghty (2), as modified by Cartland and Koch (1) for use on rats, was employed in these investigations. A few modifications of the Cartland and Koch procedure were found advantageous: (a) The tuberculin syringe used for the dye and the heart puncture needle were sterilized by boiling for several minutes in heavy

\* The experimental data in this and the following paper are taken from the dissertation submitted by James M. Orten to the Graduate School of the University of Colorado, August, 1932, in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Other material from this dissertation has been previously published.

A preliminary report of this paper was made before the Division of Biological Chemistry at the meeting of the American Chemical Society at Denver, August, 1932.

mineral oil. This procedure, besides sterilizing the instruments, left them covered with a complete film of oil which was effective in retarding the clotting time of the blood. This method was found especially helpful in obtaining successful determinations on polycythemic rats, since the blood from many of these animals clotted rapidly. (b) A different type of hematocrit tube than that described by Cartland and Koch was used. The tubes were made from 1 cc. pipettes having a bore of from 2.5 to 3.0 mm. and graduated in 0.01 cc. These were cut and ground down on a fine emery stone exactly to the 0.0 cc. graduation. They were then cut off approximately at the 0.6 cc. mark. This gave an accurate 0.5 cc. hematocrit tube, which had the distinct advantage that it could be easily cleaned since it was open at both ends. For the hematocrit determinations, the end at the 0.0 cc. graduation was covered tightly with thin rubber dam moistened on the outside, and was then inserted into a hole slightly smaller in diameter than the tube and extending about three-fourths of the way through the larger half of a No. 0 rubber stopper. This arrangement not only provided a tight seal for the hematocrit tube, but also served as a cushion for the tube during centrifugalization. (c) In contrast to the one graduated hematocrit tube used in the original method, two of the graduated tubes described above were used in each determination, so that, in the process of securing the undyed plasma used in the preparation of the color standard, the cell volume of the *original* heart blood could be determined. From this value, the cell volume and plasma volume of the *original* blood could be calculated from the total blood volume. (d) Physiological saline solution (0.85 per cent) was used exclusively as a diluent rather than distilled water, since the turbidity, which frequently occurred when the latter was used as the diluting fluid, was thereby prevented.

A speed of 1800 revolutions per minute for 30 minutes, in a centrifuge having an arm 25 cm. in diameter, was used to pack the cells in all of the hematocrit determinations. For the color standard, a suitable dilution of the 2 per cent solution of vital red used for injection into the heart was employed. A 1:1000 dilution was found suitable for the standard when the blood volume of normal rats weighing from 200 to 225 gm. was determined.

*In vitro* checks of the procedure on measured quantities of blood

yielded results well within the 4 per cent limit of accuracy claimed for the method by Cartland and Koch.

Blood volume determinations were made on all of the rats used in the previous work on cobalt polycythemia (3, 4). These animals had been continued on their respective diets, the care and feeding technique described in the previous publications being used. The ages of the animals at the time the determinations were made are given in Tables I to IV.

TABLE I  
*Blood Volume Findings in Growing Rats on Stock Diet\**

Rat No.	Weight	Blood volume		Volume per 100 gm. body weight	
		Total	Per 100 gm. body weight	Cells	Plasma
	gm.	cc.	cc.	cc.	cc.
1	184	12.5	6.78	3.34	3.44
2	294	18.2	6.15	2.92	3.23
3	222	13.7	6.15	2.92	3.23
4	296	19.1	6.42	3.05	3.37
5	220	14.8	6.64	2.59	4.05
6	297	18.5	6.20	2.95	3.25
7	310	17.8	5.78	2.69	3.09
8	252	17.5	6.92	3.11	3.81
Average ..	259	16.6	6.38	2.95	3.43

\* Determinations were made when rats were 60 to 120 days of age.

### *Results*

Table I shows the data obtained from blood volume determinations on normal, growing rats from the stock colony. These values agree with those obtained on young rats by Scott and Barcroft (5), who used a modified carbon monoxide method, and by Cartland and Koch (1).

The results of the blood volume determinations made on all of the rats used in the previous study (4) to determine the metal responsible for the polycythemia are given in Table II. Animals receiving the stock diet (Group 1) showed a lower average blood volume, expressed as cc. per 100 gm. of body weight, than that of the smaller rats reported in Table I. The finding of a decrease

# 460      Blood Volume in Cobalt Polycythemia

in blood volume per 100 gm. of body weight for larger animals is in accord with the results of Cartland and Koch (1). Control rats, receiving the basal milk-iron-copper diet (Group 2), were found to have cell, plasma, and blood volume values per 100 gm.

TABLE II

*Group Averages of Blood Volume Findings in Control Rats and Rats Fed Milk-Iron-Copper Diet Supplemented by Various Other Metals\**

Group No.	No. of rats	Supplement to milk-Fe-Cu diet	Average body weight	Blood volume		Volume per 100 gm. body weight	
				Total	Per 100 gm. body weight	Cells	Plasma
			gm.	cc.	cc.	cc.	cc.
1	7	Stock diet only	386	20.84	5.48	2.57	2.91
2	3	None	291	16.3	5.58	2.50	3.06
3	2	Mn	300	16.7	5.57	2.57	3.01
4†	2	Co	284	21.8	7.62	5.56	2.13
5	2	Ni	287	15.8	5.39	2.74	2.66
6	2	Zn	312	18.6	5.98	2.37	3.61
7	2	Mn, Co	241	21.2	8.79	6.12	2.67
8	2	“ Ni	236	15.1	6.37	3.10	3.27
9	2	“ Zn	295	18.7	6.43	3.48	2.97
10	2	Co, Ni	229	17.5	7.58	5.11	2.45
11†	2	“ Zn	235	18.1	7.70	4.65	3.05
12	2	Ni, “	282	15.8	5.63	2.45	3.18
13	2	Mn, Co, Ni	273	21.3	7.76	5.41	2.35
14	2	“ “ Zn	276	23.2	8.38	6.39	1.99
15	2	Co, Ni, “	173	11.7	6.78	3.63	3.13
16	2	Mn, “ “	288	Determinations unsuccessful			
17	2	“ Co, Ni, Zn	239	20.2	8.45	6.15	2.30
Average for non-cobalt groups (15 animals).....			286	16.7	5.85	2.74	3.11
Average for cobalt groups (14 animals).....			244	19.4	7.89	5.38	2.51

\* Group averages were obtained from determinations made when the animals were 270 to 336 days of age.

† Determinations on one rat only.

of body weight that agreed remarkably well with those of the normal controls (Group 1). Animals receiving supplements to the milk-iron-copper diet other than cobalt (Groups 3, 5, 6, 8, 9,

and 12) showed values in all determinations almost identical with those obtained from the rats on the unsupplemented basal diet (Group 2). However, all groups of animals in which cobalt was given as a supplementary metal (Groups 4, 7, 10, 11, 13-15, and 17) showed high blood and cell volumes per 100 gm. of body weight, whereas no significant alteration had occurred in plasma volume. Furthermore, Table II shows that, in spite of the relatively low average body weight of the animals of the cobalt groups, their average total blood volume was greater than that of the rats of the non-cobalt groups.

The data obtained from the blood volume determinations made on all of the rats used previously to determine the effect of cobalt

TABLE III

*Group Averages of Blood Volume Findings in Rats Fed Milk-Iron-Copper Diet Supplemented by Cobalt Sulfate\**

Group No	No. of rats	Supplement to milk-Fe-Cu diet	Average body weight	Blood volume		Volume per 100 gm. body weight	
				Total	Per 100 gm. body weight	Cells	Plasma
			gm.	cc.	cc.	cc.	cc.
1	4	None	289	16 9	5.80	2 35	3.45
2	5	CoSO <sub>4</sub>	236	18 0	7.61	4 48	3.13

\* Group averages were obtained from determinations made when the animals were 234 to 245 days of age.

sulfate feeding (4) are given in Table III. The results are identical with those found in the groups given cobalt chloride. The rats fed cobalt sulfate showed a definite increase in total blood volume, and in blood volume and cell volume per 100 gm. of body weight, over their milk-iron-copper controls. The plasma volume showed no significant alteration as the result of cobalt administration.

In Table IV are given the blood volume data for some additional control rats and for rats fed a milk-iron-copper diet supplemented by a mixture of metals. The latter belonged to the lot in which polycythemia was first observed. These animals were continued on the diets described in a preceding paper (3), with the exception noted below for Group 3-A, until the blood volume

determinations were made. The age of the rats at this time varied from 435 to 457 days. The values for the rats on the stock diet and on the milk-iron-copper diet agree well with those found for the corresponding groups given in Tables II and III. The animals of Group 3-A, once polycythemic but from whose diet cobalt had been removed 180 days previously, showed values significantly lower than those of Group 3-B in which the cobalt feeding had not been discontinued, thus demonstrating that the removal of the cobalt had resulted in a decrease of the high blood volume toward normal. A similar decrease in the hemoglobin and erythrocyte values of these animals after the removal of cobalt

TABLE IV

*Group Averages of Blood Volume Findings in Control Rats and Rats Fed Prolonged Supplemented and Unsupplemented Milk-Iron-Copper Diets\**

Group No.	No of rats	Supplement to milk-Fe-Cu diet	Average body weight	Blood volume		Volume per 100 gm. body weight	
				Total	Per 100 gm. body weight	Cells	Plasma
			gm.	cc.	cc.	cc.	cc.
1	2	Stock diet only	393	21.6	5.49	2.65	2.84
2	3	None	245	13.8	5.38	2.41	2.97
3-A	2	Mn, Ni, Zn†	250	17.9	7.12	3.39	3.73
3-B	2	“ Co, Ni, Zn	241	25.4	10.61	8.05	2.56

\* Group averages were obtained from determinations made when the animals were 435 to 457 days of age.

† Cobalt feeding was discontinued 180 days previous to the determinations. The animals had been maintained polycythemic for approximately 220 days prior to the removal of cobalt from their diet.

from the diet was reported in an earlier publication (4). The rats in which cobalt administration had been continued until they were 457 days of age (Group 3-B) showed extremely high values for total blood volume and for blood volume and cell volume per 100 gm. of body weight, whereas the plasma volume remained essentially normal. The blood volume findings of one rat of this group were of special interest, as the hemoglobin and erythrocyte count of this animal had reached exceptionally high levels. The blood volume values were correspondingly high, the blood volume per 100 gm. of body weight being 11.5 cc., of which 9.4 cc. were cell volume and 2.1 cc., plasma volume.

## CONCLUSION

The evidence from the foregoing blood volume studies warrants the conclusion that cobalt, when fed *with copper* to rats on a milk-iron diet, produces a *true* polycythemia characterized by an actual increase in the number of circulating red blood cells.

## SUMMARY

1. A definite increase in blood volume was found in rats made polycythemic by cobalt feeding.

2. The increase in blood volume was due to a rise of cell volume rather than to any significant variation of the plasma volume.

3. Cobalt supplementing a milk-iron-copper diet produces a *true* polycythemia.

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## THE EFFECT OF MANGANESE ON COBALT POLYCYTHEMIA\*

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(Received for publication, September 13, 1932)

Previous work (1-3) has shown that cobalt, when fed *with copper* to rats on a milk-iron diet, produces a polycythemia characterized by a marked increase in the hemoglobin, erythrocyte, cell volume, and blood volume values. Certain animals that had received manganese in addition to cobalt showed somewhat higher body weights and blood values than those of the cobalt-non-manganese groups. All of the polycythemic animals were continued, therefore, on their respective dietary supplements<sup>1</sup> (2), and further determinations of the hemoglobin, erythrocyte, and cell volume values were made.

Group averages of the data obtained when the animals were 255 to 282 and 395 to 402 days of age are given in Table I. It will be observed that at the earlier age the animals of the cobalt-manganese group exhibited a more pronounced polycythemia than those of the cobalt-non-manganese group. Furthermore, at 395

\* The experimental data in this and the preceding paper are taken from the dissertation submitted by James M. Orten to the Graduate School of the University of Colorado, August, 1932, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Other material from this dissertation has been previously published.

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<sup>1</sup> Attention is called to the fact that nickel and zinc have been included in the supplements to the milk-iron-copper diet of some of the animals. However, as these two metals have proved inert so far as cobalt polycythemia is concerned, the data presented in this paper are not affected by their presence.

to 402 days of age, when the animals were sacrificed, the exceedingly high cell volume and hemoglobin values still persisted in the cobalt-manganese group with only a relatively slight decrease in the erythrocyte count, whereas a decrease in all blood values toward normal had occurred in the cobalt-non-manganese group.

TABLE I

*Blood Findings (Group Averages) on Rats Made Polycythemic by Diet Containing Cobalt Both with and without Manganese*

Group	No of rats	Age	Average body weight	Hb per 100 cc.	Erythrocytes per c.mm.	Cell volume
		<i>days</i>	<i>gm.</i>	<i>gm</i>		<i>per cent</i>
Cobalt-non-manganese	6*	255-282	230	17 3	10,400,000	65
	3†	395-402	228	16 7	9,450,000	56
Cobalt-manganese	8	255-282	257	21 6	12,600,000	76
	8	395-402	261	21 5	11,600,000	74

\* Two animals of this group had died prior to these determinations.

† Three more animals of this group had died prior to these determinations, one as the result of a blood volume determination.

TABLE II

*Blood Volume Findings (Group Averages) at 270 to 336 Days of Age on Rats Made Polycythemic by Diet Containing Cobalt Both with and without Manganese*

Group	No. of rats	Average body weight	Blood volume		Volume per 100 gm body weight	
			Total	Per 100 gm body weight	Cells	Plasma
		<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
Cobalt-non-manganese.....	6	230	16 9	7 34	4.62	2.72
Cobalt-manganese.....	8	257	21 4	8 34	6 02	2.33

It is significant to note that four of the original eight animals fed cobalt without manganese had died of unknown cause prior to the time of the final blood determinations, whereas all of the rats receiving cobalt with manganese were alive even though they had a more intense polycythemia. Autopsies on the four rats of the cobalt-non-manganese group that had died without

apparent cause, and on the animals that were sacrificed at the end of the experiment, revealed no gross pathological changes. Moreover, the rats of the cobalt-manganese group were in a much better nutritive condition than those of the cobalt-non-manganese group.

Confirmatory blood and autopsy findings were obtained on another group of cobalt-non-manganese animals given cobalt sulfate instead of cobalt chloride. The same tendency for the blood values (hemoglobin, erythrocyte, and cell volume) to decrease toward normal was observed upon long continued feeding of cobalt without manganese. The death of two of the five rats of this group from unknown cause is likewise in accord with the results obtained when cobalt chloride was fed without manganese.

The fact that death occurred in 46 per cent of the animals receiving either the chloride or sulfate of cobalt without manganese indicates that this element, even when given at the low level used in our experiments (0.5 mg. of cobalt per day six times a week), exerts a slow toxic action similar to that reported for larger amounts of cobalt by Waltner and Waltner (4). As none of the rats receiving manganese in addition to cobalt succumbed, it seems probable that manganese acts either directly or indirectly to decrease this apparent toxicity of cobalt.

Average blood volume values at 270 to 336 days of age for the animals reported in Table I, compiled from the data given in the preceding paper (3), are given in Table II. The rats of the cobalt-manganese group show a higher average total blood volume, and higher average blood and cell volumes per 100 gm. of body weight, than those of the cobalt-non-manganese group. These results are in harmony with the hematological findings reported in Table I.

#### CONCLUSIONS

1. The foregoing results indicate that manganese has some stabilizing influence on the increased hemoglobin, erythrocyte, cell volume, and blood volume values characteristic of cobalt polycythemia.

2. Manganese appears to act in some manner to alleviate the toxic condition resulting from the long continued administration of small quantities of cobalt.

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## THE EFFECT OF THE PROLONGED FEEDING OF A MILK-IRON-COPPER DIET TO RATS\*

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The work of several investigators (1-3, 7) has established the fact that weanling rats on a milk diet supplemented by iron develop a severe nutritional anemia and die, whereas the addition of a trace of copper to this diet permits the animals to maintain an approximately normal hemoglobin level and to grow satisfactorily. The purpose of this report is to present the data obtained from blood studies made on rats after the prolonged feeding of an exclusive milk-iron-copper diet.

Three groups of rats, taken from the control animals of experiments previously reported (3, 4, 6) on the relation of metals to nutritional anemia and polycythemia, were studied. Erythrocyte counts, and hemoglobin and cell volume determinations, made just before the animals were sacrificed, are given in Table I, with the ages and body weights of the rats at the time they were killed. Table II gives composite averages of the three groups together with those of seven normal control rats for Group I.

The animals of Group I, after being placed in individual glass cages at the weaning age, were fed whole milk *ad libitum*, supplemented by 0.5 mg. of iron and 0.025 mg. of copper 6 days a week, and were continued on the milk-iron-copper diet until they were 346 to 395 days of age. The body weights then averaged 317 gm., which is definitely lower than that of their normal controls on a stock diet. All of the rats showed erythrocyte counts and hemo-

\* A preliminary report of this paper was made before the Division of Biological Chemistry at the meeting of the American Chemical Society at Denver, August, 1932.

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globin and cell volume values only slightly lower than those of the control animals. As reported in another paper (5), total blood volume determinations, made on this group at about 300 days of age, averaged 5.58 cc. per 100 gm. of body weight, a normal value

TABLE I

*Blood Findings in Rats Fed Milk-Iron-Copper Diet for Prolonged Periods*

Group No.	Rat No.	Age	Body weight	Hemo- globin	Erythrocytes per c.mm.	Cell volume
		<i>days</i>	<i>gm.</i>	<i>gm.</i>		<i>per cent</i>
I	124	346	364	15.3	8,125,000	52
	125	346	338	15.1	9,075,000	50
	122	395	254	13.4	7,070,000	45
	121	395	283	13.2	7,370,000	43
	105	395	344	13.5	7,810,000	56
II	69	450	237	15.0	6,650,000	51
	70	450	199	15.2	8,000,000	50
	71	454	286	13.2	8,050,000	Lost
	73	454	249	15.6	8,950,000	43
	74	454	296	14.5	9,300,000	45
III	13	667	263	13.9	8,700,000	
	15	667	283	14.0	8,500,000	

TABLE II

*Composite Averages of Data from Table I and from Group of Seven Normal Control Rats for Group I*

Group No.	Age	Average body weight	Hemo- globin	Erythrocytes per c.mm.	Cell volume
	<i>days</i>	<i>gm.</i>	<i>gm.</i>		<i>per cent</i>
Normal controls (of Group I)	395	437	15.0	9,020,000	51
I	346-395	317	14.1	7,886,000	49
II	450-454	253	14.7	8,190,000	47
III	667	273	14.0	8,600,000	

for rats of this age. Autopsies on the animals of this group revealed no gross abnormalities.

The animals of Group II were likewise kept on the milk-iron-copper diet from the time of weaning until they were from 450 to 454 days of age. The body weights of these animals were also

found to be somewhat lower than those of normal stock rats of comparable age. The hemoglobin, erythrocyte, and cell volume values were almost identical with those found in Group I. On autopsy, no gross abnormalities were observed. Histological examination of liver, lungs, heart, spleen, kidneys, stomach, testes, and bone marrow of Rats 70 and 71 showed no pathological changes. Successful total blood volume determinations made on Rats 70, 71, and 73 averaged 5.38 cc. per 100 gm. of body weight, which agrees with the normal figure (5).

The animals of Group III, at the time of weaning, were placed on an exclusive whole milk diet until a severe nutritional anemia developed (6), when supplements of iron and copper were added 6 days a week (Rat 15 received 0.05 mg. of copper; Rat 13, 0.025 mg. of copper). Both showed a prompt recovery from the anemia, and were continued on the milk-iron-copper diet until they were 667 days of age. Even though the animals were approximately 11 and 7 months older than the rats of Groups I and II, respectively, the blood findings agree with those found for these two groups. Routine gross and histological examinations of tissues obtained at autopsy revealed no pathological changes.

In general, all of the animals exhibited an average amount of reserve fat, and appeared to be in good nutritive condition. The coats of the younger animals were fine and smooth; however, those of the older animals were coarse and somewhat shaggy, as is often the case in old normal rats. The comparatively low body weights may well have been due to the low caloric intake resulting from the exclusive liquid diet.

Since the possibility of coprophagy has not been entirely eliminated, we cannot regard these results as final evidence for the completeness of an exclusive milk-iron-copper diet for rats. However, the data indicate that milk supplemented by iron and copper provides a diet which permits rats to maintain an approximately normal blood picture, and to live for long periods of time in an apparently normal state of health.

#### SUMMARY

Data are presented showing that an exclusive milk-iron-copper diet will maintain rats in an apparently normal condition for prolonged periods of time.

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## COBALT IN ANIMAL NUTRITION\*

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The biological significance of cobalt has attracted the attention of relatively few investigators. McHargue (1) studied the occurrence of certain inorganic elements including cobalt in soils, plants, and animals, but the only definite values reported for cobalt were a trace in blue-grass, soy bean leaves, soy bean seeds, and 1.5 parts per million in Kentucky virgin soil. Fox and Romage (2) from spectrographic observations of the tissues of *Archidoris tuberculata* report the presence of 0.0003 per cent cobalt in the liver, and of smaller amounts in certain other tissues of polychaetes. Dutoit and Zbinden (3) in a spectrographic analysis of the ashes of human organs observed cobalt in the pancreas and traces of cobalt in some of the other organs, but none in the liver.

Bertrand and coworkers are the only investigators, so far as we know, who have studied cobalt in an extensive manner. Bertrand and Mokragnatz (4) in studies on the zinc content of certain French soils encountered small amounts of cobalt in several of the soil samples. This led to a more detailed study of the cobalt content of soils and also of the cobalt content of plants (Bertrand and Mokragnatz (5)) and animals (Bertrand and Mâcheboeuf (6, 7)). Bertrand (8) was impressed with the amount of cobalt observed in the pancreas in comparison to other organs and concluded that cobalt may somehow be connected with the elaboration of insulin. In experiments on the influence

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of cobalt on the action exercised by insulin in rabbits (9), and later in dogs (10), Bertrand and Mâcheboeuf concluded that cobalt increases the hypoglycemic power of insulin. Bertrand and Nakamura (11) have conducted a few feeding experiments with mice in an attempt to study the physiological importance of cobalt. It is impossible to reach any conclusion from this work because the animals were placed on diets deficient in factors other than cobalt and survived only about 3 weeks.

Mokragnatz (12) studied the effect of cobalt in the development of cultures of *Aspergillus niger* but observed no beneficial effect. He found that in concentrations of cobalt greater than 2 parts per million growth was retarded, and in concentrations of 400 parts per million growth was completely inhibited.

The recent reports from several laboratories concerning polycythemia produced by cobalt have revived interest in the biochemical importance of this element. Waltner and Waltner (13) observed in a study of the toxicity of various metals that cobalt when added to the diet of rats produced a marked increase in the number of red blood cells. An increase in erythrocytes due to the feeding of cobalt to dogs has been reported by Mascherpa (14).

Myers, Beard, and Barnes (15), and more recently Orten, Underhill, Mugrage, and Lewis (16), in studies on hemoglobin regeneration, reported a polycythemia produced by the addition of cobalt to a milk-iron-copper diet. Similar effects have also been observed in this laboratory (17).

These results led us to a study of the cobalt content of normal rats and those in which a polycythemia had been produced by cobalt feeding. Later we studied the distribution of cobalt in the organs of pigs which had received cobalt in their diet. These studies necessitated the development of a method for the estimation of small amounts of cobalt.

We present in this paper, first, a rapid, convenient, and accurate colorimetric method for the estimation of cobalt in biological materials which contain amounts of cobalt ranging from 0.01 to 0.5 mg. per sample; secondly, data showing that if cobalt does occur in the normal animal organism it does so in extremely minute amounts; and, thirdly, data revealing the distribution of cobalt in the bodies of rats and pigs fed a diet containing cobalt.

*Method*

Previous workers have utilized gravimetric methods designed for the general macro inorganic determination of cobalt. In the usual chemical reactions cobalt behaves quite like nickel, and in the ordinary gravimetric or volumetric analysis the problem is to separate the nickel and cobalt. Nickel can be effectively removed by precipitation with dimethylglyoxime and the cobalt remaining in the filtrate may be determined by precipitation as cobaltinitrite in acetic acid solution. Bertrand, in his early work on the cobalt content of soils and plants, used the potassium nitrite method. In his later work with Mâcheboeuf he used a colorimetric method as follows (7): The mother liquor after the precipitation and separation of nickel (Ni precipitated by dimethylglyoxime) was evaporated to dryness and heated to dull redness in a muffle furnace. The residue was taken up in hot concentrated hydrochloric acid and the solution evaporated to dryness and taken up in water. To this was added 0.2 cc. of a 1 per cent alcoholic solution of dimethylglyoxime and 2 drops of 10 per cent ammonia. The cobalt was estimated colorimetrically in the resulting brownish yellow solution; sensitivity was reported as 0.01 mg.

When nitroso- $\beta$ -naphthol is added to a hydrochloric acid solution of cobalt, a precipitate of cobaltinitroso- $\beta$ -naphthol is formed; nickel, if present, remains in solution. McHargue used this method in all of his work.

The methods mentioned above, or slight modifications of them, are neither convenient nor readily adaptable for the estimation of small amounts of cobalt in biological materials, since they all require the removal of iron, copper, and certain other elements. The precipitations required may involve loss of cobalt through co-precipitation, and for small amounts of cobalt the solubility factor of cobaltinitrite becomes an important factor. In the case of the nitroso- $\beta$ -naphthol method, often a small amount of nitroso-naphthol is formed which is not dissolved by boiling in nitric acid, and which may obscure any color due to cobalt.

We have developed a convenient and simple colorimetric method for the estimation of small amounts of cobalt based upon the observation recorded by Van Klooster (18) that the nitroso derivative of R salt, sodium-2,3,6- $\beta$ -naphtholdisulfonate, when treated

with cobalt salts forms a red dye of the composition of  $(C_{10}H_5NO_8S_2Na_2)_3Co$ . The color formed is exceedingly stable and is not destroyed by heating with acids. Other cations such as Fe, Ni, Cu, and Zn, form colored dyes with the R salt but these colors are destroyed by boiling with an excess of nitric acid. The color is best developed in a solution made slightly alkaline with sodium acetate, and then by adding nitric acid and boiling, the red color formed by cobalt is produced.

*Reagents*

*Standard Cobalt Solution*—1 cc. = 0.1 mg. of cobalt. A solution of pure  $CoCl_2 \cdot 6H_2O$  (0.2017 gm. per 500 cc.) in redistilled water.

*Indicator Solution*—1 gm. of the nitroso R salt<sup>1</sup> made up to 100 cc. with redistilled water.

C. P. sodium acetate.

Saturated solution of potassium hydroxide.

Hydrochloric acid.

Nitric acid.

*Procedure*—Samples containing preferably between 0.05 and 0.5 mg. of cobalt are thoroughly ashed at dull red heat and the ash taken up in a small amount of 1:1 hydrochloric acid. To this solution, whose volume for convenience should not be more than 20 to 25 cc., are added 2 drops of phenolphthalein, 2 cc. of indicator reagent, and about 2 gm. of sodium acetate. The mixture is warmed to about 70° and thoroughly stirred. Then while the stirring is continued, saturated potassium hydroxide is added slowly, drop by drop, until the reaction medium is just alkaline, as indicated by the phenolphthalein. The contents of the beaker are then heated to boiling and concentrated nitric acid added drop by drop until there is a distinct excess of the acid; the boiling is continued for 1 to 2 minutes. A permanent change in color toward red indicates the presence of cobalt. The colors produced by iron, nickel, and other metal salts are destroyed by oxidation with nitric acid. The solution is allowed to cool and made up in a 50 cc. volumetric flask with distilled water. This is compared in a colorimeter against a standard cobalt solution containing a somewhat similar concentration of cobalt and prepared in an analogous manner. By this procedure we have been able to determine as little as 0.01 mg. of cobalt.

<sup>1</sup> Obtained from Eastman Kodak Company.

Results are presented in Table I to show the recovery of cobalt through this method. All the samples used in this study had been analyzed previously and found to contain less than 0.01 mg.

TABLE I  
*Recovery of Cobalt Added to Biological Materials before Ashing*

Material	Weight of sample	Co added	Co found	Recovery
	gm	mg	mg	per cent
Beef liver	5	0 2	0 195	97 5
" "	5	0 3	0 3	100
Lobster	5	0 2	0 194	97
Dog liver	5	0 2	0 194	97
Beef "	5	0 5	0 495	99
" "	5	0 05	0 05	100

TABLE II  
*Effect of Other Cations on Recovery of Cobalt*

Solution of distilled water containing	Amount of indicator added	Co recovered		Solution of distilled water containing	Amount of indicator added	Co recovered	
		mg	per cent			mg	per cent
mg	cc			mg	cc		
0 2 Co	2	0 2	100	0 3 Co	2	0 2	100
				5 0 Mn			
0 2 Co	2	0 2	100				
1 0 Ni				0 2 Co	2	0 2	100
				5 0 Zn			
0 2 Co	2	0 2	100				
5 0 Ni				0 2 Co	2	0 2	100
				0 5 Fe			
0 2 Co	2	0 15	75	0 5 Cu			
10 0 Ni							
0 2 Co	4	0 2	100	0 2 Co	2	0 195	97 5
10 0 Ni				0 5 Mn			
				0 5 Zn			
0 2 Co	2	0 2	100	0 5 Fe			
5 0 Cu				0 5 Cu			

of Co per sample used (see Table III). It is readily seen that added cobalt can be quantitatively recovered from biological materials by this method.

As was previously mentioned, the nitroso R salt forms colored

compounds with other cations besides cobalt, but on boiling with an excess of nitric acid these colors are destroyed and only the red color of cobalt remains. To insure complete recovery of cobalt one must have an excess of indicator so that if other cations are present there will be sufficient indicator to unite with the cobalt. Table II demonstrates the recovery of cobalt in solutions containing known amounts of other cations. It is observed from Table II that cobalt was quantitatively recovered in the presence of nickel, manganese, zinc, iron, and copper. When other elements are present in relatively large amounts, it is necessary to increase the amount of R salt added. For example, 2 cc. of indicator added to a solution of 0.2 mg. of cobalt and 10 mg. of

TABLE III  
*Cobalt Analysis of a Few Biological Products*

Each sample contained less than 0.01 mg. of cobalt.

Material	Weight of sample	Material	Weight of sample
	gm.		gm
Rat liver	1.984	Beef liver	5.0
" "	2.073	Canned peas	5.0
Abbott's liver extract	5.0	Milk	100.0
Lobster	5.0	Yeast	10.0
Dog liver	5.0	Lettuce	20.0

nickel gave only a 75 per cent recovery; however, when 4 cc. of indicator were used, recovery of cobalt was complete.

A few precautions should be taken to insure the best results. Ashing should be done in platinum dishes and at a temperature not exceeding 500°. The addition of calcium carbonate to materials which ash with difficulty decreases the time of ashing. The reaction of the nitroso R salt with cobalt takes place most readily and most thoroughly in the presence of sodium acetate and at a slightly alkaline pH; thus one should slowly make the solution alkaline, with the indicator present and with constant stirring to insure thorough mixing of the indicator with the solution. Blank determinations should be run frequently to check any possible source of contamination.

The results obtained when this method was applied to a few

biological substances are given in Table III. It is observed that cobalt, if it occurs in biological substances, does so in extremely small amounts, amounts less than 0.01 mg. for the weight of sample used. At present, efforts are being made to increase the sensitivity of the method so that we may determine smaller amounts of cobalt.

Since we have been unable to detect cobalt in the animal organs studied, and since it is known that cobalt when administered to rats produces a polycythemia, we were interested in determining whether or not we could detect cobalt in the body of an animal which had been fed cobalt, and if so in what tissues the cobalt was distributed.

### *Animal Studies*

For the analysis of the total body, rats were used that had been employed in polycythemia studies. All these rats were fed a basal diet consisting of milk fortified with iron, copper, and manganese in amounts sufficient to supply each rat 0.5 mg. of Fe, 0.05 mg. of Cu, and 0.04 mg. of Mn per day. Some of the rats remained on the basal ration alone, others were given cobalt varying in amount from 0.1 to 2.0 mg. of Co per rat, daily. When the polycythemia studies were concluded, the rats were killed without loss of blood and the digestive system removed.

Table IV includes the data obtained by the analysis of the whole bodies and digestive systems of these rats, and the amount of cobalt which each rat received. In no case was cobalt definitely detected in concentrations above 0.01 mg. in the body or digestive tract of a rat not receiving added cobalt, and in each case it was detected in those rats receiving added cobalt. The amount of cobalt retained in the body is proportional to the amount of cobalt administered in the diet. For example, Rat 594 received 0.6 mg. of cobalt per day and the amount of cobalt found in its body was 0.150 mg. Rat 601 received 1.0 mg. of cobalt per day and the amount detected in its body was 0.208 mg.

With young rats receiving more than 0.6 mg. of cobalt per day definite toxic effects were observed. At levels of 1.0 and 1.5 mg. per day the rats rapidly lost weight, and at 2.0 mg. per day the rats survived only for a period of 2 weeks. Rat 606, receiving 2.0 mg. of cobalt per day, was very feeble at the time it was taken for

analysis, and the amount of cobalt found in the entire body was 0.290 mg. With older rats no toxic effects were observed at levels less than 1.0 mg. per day.

For the study of the distribution of cobalt in the tissue, pigs were utilized because they could be fed larger amounts of cobalt and the organs would offer larger samples for analysis. Four pigs were used and given only whole cow's milk *ad libitum*, to

TABLE IV  
*Analysis of Rats Administered Cobalt and Amount of Cobalt Fed*

Rat No	Age when placed on experiment	Time on experiment	Co fed per day	Weight when killed	R b c when killed	Co in body	Weight of digestive system	Co in digestive system
	<i>wks.</i>	<i>wks.</i>	<i>mg</i>	<i>gm.</i>	<i>millions per c.mm</i>	<i>mg</i>	<i>gm.</i>	<i>mg</i>
351	3	10	0 0		7 8	<0 01		
352	3	10	0 5		12 6	0 0726		
341	3	13	0 0	140	7 4	<0 01		
340	3	13	0 5	93	11 5	0 0645		
434	8-10	6	0 0	280	8 7	<0 01		
437	8-10	6	1 0	173	10 9	0 146		
450	3	18	0 0	258	8 9	<0 01	30 0	<0 01
451	3	18	0 1	278	11 1	0 0484	31 0	0 125
452	3	18	0 0	218	9 0	<0 01	28 0	<0 01
453	3	18	0 1	210	13 3	0 0517	35 0	0 122
454	3	18	0 1	149	14 9	0 039	20 0	0 101
594	3	3	0 6	34		0 150	5 0	0 129
595	3	3	0 6	42		0 105	5 0	0 0715
601	3	3	1 0	37		0 208	6 0	0 102
604	3	2	1 5	40		0 222	6 0	0 29
606	3	2	2 0	28		0 290	3 0	0 067

which were added daily 25 mg. of Fe, 5 mg. of Mn, and 2 mg. of Cu. Two of the pigs received 25 mg. of cobalt in addition. The pigs were 2 weeks old when started on the experiment; one from each diet was killed at the age of 7 weeks, and the remaining two were killed at 12 weeks.

Results are presented in Table V which show the distribution of cobalt in the tissues of pigs that have received cobalt in their diet, and the relative absence of cobalt in similar tissues of pigs that have not received cobalt. It is noted that cobalt is distributed to many tissues of the pig with the greatest amount found

in the liver, pancreas, and spleen. The vertebræ and ribs contained 1.98 mg. of cobalt per kilo of dry material, whereas the long bones contained an amount undetectable. It may be that the vertebræ and ribs are more active in erythrocyte formation and this may be where cobalt exerts its effect in producing a polycythemia.

We have stated that samples of tissues from animals not subjected to cobalt treatment were found to contain less than 0.01 mg. of Co

TABLE V

*Analysis of Certain Tissues of Pigs on a Basal and Basal Plus Cobalt Diet*

	Co-fed pig	Normal control	Co-fed pig	Normal control
Pig No	122	125	123	121
Age when placed on experiment, <i>wks</i>	2	2	2	2
Time on experiment, <i>wks</i>	5	5	10	10
Co fed per day, <i>mg.</i>	25 0	0 0	25 0	0 0
R.b c at beginning of experiment, <i>millions per c mm.</i>	4 3	5 7	5 1	5 5
R.b c at end of experiment, <i>mil-</i> <i>lions per c mm.</i>	12 9	8 2	10 1	9 4
Co per 10 gm (dry), <i>mg</i>				
Liver	0 084	<0 01	0 0318	<0 01
Pancreas	0 080	<0 01		
Spleen	0 050	<0 01	0 025	<0 01
Heart	0 0397	<0 01		
Blood	0 0382	<0 01		
Vertebræ and ribs	0 0198	<0 01		
Skin	0 011	<0 01		
Muscle	0 009	<0 01		
Long bones	<0 01	<0 01		
Lung			0 0297	<0 01

per sample. However, a color sufficient to indicate a trace of cobalt was never observed in the analysis of any of these tissues. It is impossible for us to state that normal animals do not contain cobalt and that this element is unnecessary for normal development, but when a normal rat weighing 250 gm. contains much less than 0.01 mg. of cobalt it must be concluded that if this metal is essential it is active in extremely small amounts.

That cobalt in traces so small as to be undetected by the method described in this paper may be active physiologically is indicated

by the small amount necessary to produce polycythemia. The presence of 0.04 to 0.05 mg. of Co in the entire body of a rat is sufficient to produce a decided polycythemia (see Rat 454, Table IV). The results obtained for the cobalt content of the different tissues in cobalt-fed pigs indicate that this element does not concentrate in any particular organ. If the polycythemia is produced by the action of cobalt in a specific organ or tissue the amount active in producing the condition would be still less.

#### SUMMARY

1. A method for the estimation of cobalt in biological materials has been outlined. It is applicable to samples containing 0.01 to 0.5 mg. of cobalt.

2. The results obtained when this method was applied to a few biological substances are given.

3. The entire body of rats fed a milk, iron, copper, and manganese diet was found to contain less than 0.01 mg. of cobalt. Definite amounts of cobalt were found in all rats fed a similar diet plus cobalt, and the quantity present was proportional to the amount fed. The presence of 0.04 to 0.05 mg. of cobalt in the entire body of a rat was sufficient to produce a decided polycythemia.

4. No cobalt was detected in tissues taken from pigs fed a milk, iron, copper, and manganese diet, but definite quantities were detected in practically all the tissues taken from pigs fed a similar diet plus cobalt.

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## THE *o*-QUINONE TEST FOR CYSTEINE

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A simple qualitative test for cysteine, which involved the use of *o*-benzoquinone, was described in a previous paper (1). This reaction, which resulted in the formation of a red-colored complex soluble in chloroform, was given only by cysteine, among the group of compounds tested. In the use of a quinone this test was comparable to the highly specific  $\beta$ -naphthoquinone-sodium sulfonate reaction for cysteine which has been the subject of extensive research by Sullivan (2). Another test for cysteine depending on the reaction of a quinone with this amino acid was described by Fleming (3), who used as the reagent dimethyl-*p*-phenylenediamine hydrochloride in the presence of an oxidizing agent.

Because of the simplicity of the technique involved in the *o*-benzoquinone test, the investigation of this reaction has been continued. An additional group of substances has been studied in order to ascertain what molecular groups are necessary to give this coloration. An improved method of preparing the quinone has also been developed and a direct study of the chemistry of the cysteine-quinone complex is in progress.

*Preparation of o-Benzoquinone Solution*—The original method (1) of preparing the quinone, based on the procedure of Willstätter and his coworkers (4), has been improved chiefly by the use of a cooling mixture composed of solid carbon dioxide and alcohol (5). A mixture of 0.5 gm. of powdered catechol, 1.2 gm. of dried silver oxide (4), 2 gm. of anhydrous sodium sulfate, and 15 cc. of dry ether was placed in a test-tube previously chilled in the cooling mixture. The tube was then removed from the cooling bath, shaken vigorously for 1 to 2 minutes, and the mixture filtered

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quickly. The ether filtrate, when gradually cooled to  $-70^{\circ}$  deposited red crystals of the quinone. If the cooling was too rapid, the colorless, more colloidal form was obtained (5). The crystals were freed from the solution by decantation, washed twice with 1 cc. of cold ether, and dissolved in 20 cc. of dry cold chloroform. When 4 cc. of the greenish tan-colored solution which resulted were shaken for 2 to 3 minutes with 2 cc. of a 0.1 per cent aqueous solution of cysteine hydrochloride, a cherry-red color was developed in the chloroform layer.

The use of other oxidizing agents, such as lead oxide, or hydrogen peroxide with peroxidase, proved to be unsuccessful for the preparation of the quinone.

*Reaction of Various Compounds with o-Quinone*—About 2 cc. of a dilute aqueous solution (approximately 0.1 per cent if possible) of the substance to be tested were shaken for 2 to 3 minutes with 3 or 4 cc. of a cold chloroform solution of the quinone prepared by the procedure described above. In cases in which the material was more soluble in weak acids than in water, a dilute hydrochloric acid solution was used, since the cysteine reaction was not affected by such a medium ((1) p. 484). If the substance was soluble in chloroform, but not in water, an aqueous suspension of the material was treated with a chloroform solution of the quinone as usual.

All of the compounds listed in Table I, with the exception of the last three, gave no immediate coloration aside from the pale yellow produced by shaking a chloroform solution of o-quinone with water alone. In each case a faint pink or brown color produced in the aqueous layer after the course of several hours signified only the decomposition of excess quinone.

The last three substances, phenylhydrazine hydrochloride, hydroxylamine hydrochloride, and pyrogallol, produced with o-quinone a bright yellow coloration in the chloroform layer. This color was distinctly different from the cherry-red characteristic of cysteine, and did not interfere with the development of the latter when mixtures of each of these compounds with cysteine were treated with an excess of the o-quinone reagent.

In Table II are shown the compounds which, with the o-quinone reagent, gave a red coloration in the *aqueous*, but not in the *chloroform* layers, and thus differed from cysteine. Although these substances are closely related to cysteine, the colored com-

TABLE I  
Compounds Producing No Red Coloration with *o*-Quinone

Sample No.	Derivatives of cystine, cysteine, and related compounds		
1	Dibenzoylcystine*	$(-\text{SCH}_2\text{CH}(\text{COOH}) \cdot \text{NHCOC}_6\text{H}_5)_2$	
2	Diformylcystine*	$(-\text{SCH}_2\text{CH}(\text{COOH}) \cdot \text{NHCHO})_2$	
3	Dialanylcystine*	$(-\text{SCH}_2\text{CH}(\text{COOH}) \cdot \text{NHCOCH}(\text{NH}_2)\text{CH}_3)_2$	
4	Dibenzylidene cystine, barium salt*	$(-\text{SCH}_2\text{CH}(\text{COOBa}\frac{1}{2})\text{N}=\text{CHC}_6\text{H}_5)_2$	
5	Diguanidocystine†	$(-\text{SCH}_2\text{CH}(\text{COOH})\text{NHC}(=\text{NH})\text{NH}_2)_2$	
6	Diphthalimidocystine†	$(-\text{SCH}_2\text{CH}(\text{COOH})\text{N} \begin{array}{c} \diagup \text{CO} \diagdown \\ \diagdown \text{CO} \diagup \end{array} \text{C}_6\text{H}_4)_2$	
7	Cystine amine hydrochloride†	$(-\text{SCH}_2\text{CH}_2\text{NH}_2 \cdot \text{HCl})_2$	
8	Disulfide of thiotyrosine†	$(-\text{SC}_6\text{H}_4\text{CH}_2\text{CH}(\text{COOH})\text{NH}_2)_2$	
9	N-Formylcysteine*	$\text{HS} \cdot \text{CH}_2\text{CH}(\text{COOH})\text{NH} \cdot \text{CHO}$	
10	Cysteine-S-sodium sulfonate*	$\text{NaSO}_3 \cdot \text{SCH}_2\text{CH}(\text{COOH})\text{NH}_2$	
11	Methionine†	$\text{CH}_3\text{S} \cdot \text{CH}_2\text{CH}_2\text{CH}(\text{COOH})\text{NH}_2$	
Sample No.	Miscellaneous compounds	Sample No.	Miscellaneous compounds
12	Thiophene	20	Uric acid
13	Furfural	21	Phenol
14	Phenylisothiocyanate	22	Paraldehyde
15	Potassium ethyl xanthogenate	23	Formaldehyde
16	Sodium diethyl dithiocarbamate	24	Acetone
17	Tyramine hydrochloride	25	Hydrazine hydrate
18	Piperidine	26	Phenylhydrazine hydrochloride
19	Methylamine	27	Hydroxylamine hydrochloride
		28	Pyrogallol

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plexes produced by them possessed different solubilities from the one given by cysteine. Even isocysteine was unlike cysteine in its behavior toward *o*-benzoquinone. A comparable dissimilarity in the reaction of the two isomers toward  $\beta$ -naphthoquinone-sodium sulfonate was observed by Sullivan and Hess (6).

The isocysteine was obtained by Gabriel's synthesis (7) from dihydrouracil which was prepared according to the method of Brown and Johnson (8). The amidomercaptoethane was synthesized from bromoethylphthalimide according to Gabriel's directions (9). The thiotyrosine hydrochloride was obtained by

TABLE II  
*Compounds Producing a Coloration in the Aqueous Layer*

Sample No.	Compound	Formula	Color in aqueous layer
29	Isocysteine hydrochloride	$\text{HS} \cdot \text{CH}(\text{COOH})\text{CH}_2\text{NH}_2 \cdot \text{HCl}$	Deep red
30	$\alpha$ -Mercapto- $\beta$ -amidoethane hydrochloride	$\text{HS} \cdot \text{CH}_2\text{CH}_2 \cdot \text{NH}_2 \cdot \text{HCl}$	Magenta-red
31	N-Guanidocysteine*	$\text{HS} \cdot \text{CH}_2\text{CH}(\text{COOH})\text{NHC}(=\text{NH})\text{NH}_2$	Deep red
32	N-Benzene sulfonylcysteine*	$\text{HS} \cdot \text{CH}_2(\text{COOH})\text{NH} \cdot \text{SO}_2\text{C}_6\text{H}_5$	Magenta-red
33	Thiotyrosine hydrochloride	$\text{HS} \cdot \text{C}_6\text{H}_4 \cdot \text{CH}_2\text{CH}(\text{COOH})\text{NH}_2 \cdot \text{HCl}$	Orange-red

\* We are grateful to Professor Hans T. Clarke for his cooperation in furnishing these cysteine derivatives.

reduction of the corresponding disulfide, as described by Johnson and Brautlecht (10).

Table III shows the compounds which gave a red coloration in the *chloroform* layer. The difficulty caused by piperidine and methylamine was avoided by neutralizing the bases with 0.2 N hydrochloric acid, since the hydrochlorides of these bases gave no coloration with the quinone (*cf.* Table I). The presence of 0.2 N HCl has repeatedly been shown to have no effect on the reaction of cysteine with *o*-quinone.

The coloration produced by the esters of cysteine could be distinguished from the cysteine color by comparative tests.

When a 0.1 per cent aqueous solution of either the methyl or ethyl ester of cysteine hydrochloride was shaken with a chloroform solution of *o*-quinone, an orange-pink coloration was produced at once in the chloroform layer. After the shaking had been continued for 1 or 2 minutes, the chloroform layer became orange, then yellow. This change was different from the behavior of cysteine under similar conditions, since in the latter case the red color slowly formed in the chloroform layer was gradually deepened by further shaking to give a stable cherry-red. In this connection it is of interest to note that cystine dimethyl ester dihydrochloride has been found to give a positive Sullivan reaction for cystine,

TABLE III  
*Compounds Producing a Red Coloration in the Chloroform Layer*

Sample No.	
34	Cysteine hydrochloride.
35	“ methyl ester hydrochloride
36	“ ethyl “ “
37	Piperidine
38	Methylamine
39	Benzidine
40	Aniline*

\* The dye produced by the reaction of aniline with *o*-quinone has been studied by Jackson and Koch (11).

but the coloration produced appeared to differ in stability and intensity from that given by the acid hydrochloride (12).

The ethyl ester hydrochloride, prepared by esterification of pure cysteine, and purified as described by Cherbuliez and Plattner (13), melted at 118°. The methyl ester hydrochloride, prepared and purified by Abderhalden's method (14), melted at 135°.

A consideration of Tables I to III shows that cysteine, in aqueous hydrochloric acid solution, may be distinguished from all the other compounds tested except aniline and benzidine by means of the *o*-quinone reaction. This reaction, therefore, provides a means of distinguishing cysteine from many related nitrogenous and sulfur-containing compounds.

*Preliminary Study of Chemistry of Cysteine-o-Quinone Reaction—*  
In order to discover the source of the coloration produced, the

effect of solvents on the reaction was studied, and an attempt was made to isolate the products formed. Since the red coloration was obtained both with ordinary chloroform and with the purest chloroform available, Kahlbaum material with specified refractive index ( $n_D^{20} = 1.4450^\circ$ ), no impurity in the chloroform was responsible for the reaction. Other solvents which were non-miscible with water, *e.g.* bromobenzene, chlorobenzene, ethyl bromide, anisole, ethyl acetoacetate, and benzene, when purified by the usual methods and freshly distilled, gave yellow or orange solutions of the quinone which became red when shaken with aqueous solutions of cysteine hydrochloride. It was indicated in a previous paper ((1) p. 488) that no coloration was produced in benzene solution. It has now been found that with sufficiently pure benzene a red color is obtained in the benzene layer. Similarly, carbon tetrachloride, when free from chlorine and freshly distilled, gave a red color in the solvent layer. It therefore appears that the reaction of o-quinone with cysteine to form a colored compound is not limited to chloroform and bromoform solutions.

An investigation of the products formed from the reaction of o-quinone with cysteine hydrochloride has led to the isolation, from the chloroform layer, of a brown substance containing sulfur and nitrogen, and to the separation of catechol from the aqueous layer. The study of the pigmented sulfur-containing product is being continued and will be reported in a later publication.

The formation of catechol has been demonstrated by qualitative tests and by isolation of the crystalline solid. A sample of the crystalline quinone was filtered by suction, washed thoroughly with cold ether to remove unaltered catechol, and dissolved in chloroform. A portion of this chloroform solution was shaken with water, and the aqueous layer separated and tested for catechol with Quastel's molybdate reagent (15). No coloration was obtained, indicating the absence of catechol. When the chloroform solution of the quinone was shaken with a dilute solution of cysteine hydrochloride, and the aqueous layer separated and treated as before, the red-brown color obtained showed the presence of catechol.

Crystals of catechol were obtained by similar treatment of larger amounts of cysteine; *e.g.*, 10 cc. of a 1 per cent aqueous solution. The separated aqueous layer was acidified, and the catechol

extracted with ether. It was identified by the precipitation of an insoluble lead salt, by a positive molybdate reaction, and by a mixed melting point with a known specimen.

In connection with the formation of catechol from this reaction it should be noted that an excess of quinone is necessary to produce the characteristic red coloration. When a cysteine hydrochloride solution was treated with an insufficient quantity of *o*-quinone, only a pale yellow color was produced in the chloroform layer. The aqueous layer from this reaction, however, gave a definite positive test for catechol. The addition of more quinone produced the usual red color in the chloroform layer. It therefore appears that the initial stage of the reaction between cysteine and *o*-quinone involves the reduction of the quinone to form catechol. Nevertheless, neither a mixture of cysteine and catechol, nor a mixture of cystine, catechol, and *o*-quinone produces a coloration.

#### SUMMARY

A further study of the *o*-quinone test for cysteine has shown that this reaction may be used to distinguish cysteine from a large number of related nitrogenous and sulfur-containing substances. The test should be carried out in weak hydrochloric acid solution.

Two products have been isolated from the reaction of cysteine with *o*-quinone: catechol and a red-brown substance containing sulfur and nitrogen which is under investigation at present. The formation of the characteristic red compound is not affected by changing the solvent.

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## CHANGES IN HEXOSEPHOSPHATE, GLYCOGEN, AND LACTIC ACID DURING CONTRACTION AND RECOVERY OF MAMMALIAN MUSCLE\*

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The following chemical changes are known to occur during a short tetanic stimulation of muscle: a splitting of phosphocreatine and glycogen and a deamination and partial dephosphorylation of adenosinetriphosphate. Each of these changes is reversible, in part, during the contraction itself, so that demonstrable changes in the quantity of these constituents during or shortly after the contraction always represent the algebraic sum of cleavage and restitution. This is particularly true of phosphocreatine. In the first few seconds of tetanic stimulation the phosphocreatine diminishes rapidly, because the breakdown greatly exceeds the restitution, but as the stimulation continues, the phosphocreatine diminishes very slowly, not because less is broken down (as shown by an undiminished muscular tension) but apparently because resynthesis proceeds at an increased rate. The resynthesis can be brought about by lactic acid formation and can thus take place under anaerobic conditions. This is the interpretation to be given to the experiments of Eggleton and Eggleton (1) and of Nachmansohn (2) in the light of the observation of Lundsgaard (3) that phosphocreatine is eventually broken down completely during muscular contraction with inhibited lactic acid formation.

The restitution of glycogen, which depends on oxidative energy, is a relatively slow process and hence it should be possible to determine fairly accurately how much glycogen is broken down during contraction, but the matter is complicated by the fact

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that the breakdown of glycogen apparently does not coincide with the contraction. It was first shown by Embden and his collaborators (4) and later confirmed by Meyerhof and Schulz (5) that after a short tetanic stimulation (5 to 10 seconds) up to 50 per cent of the total amount of lactic acid formed may appear in the first few minutes after the contraction. Since, as far as is known, glycogen is the principal if not the only source of lactic acid in muscle, it would seem probable that glycogen breakdown outlasts the contraction process for a considerable period of time. This could be explained by assuming that enzyme activity, once it is accelerated by the nerve impulse, does not revert immediately to the resting state, but continues for some time at an increased rate after the nerve impulse has ceased.

The problem under discussion, *i.e.* the mechanism of lactic acid formation after the contraction, assumes a somewhat different aspect, if it is remembered that Eggleton and Eggleton (6), Davenport and Sacks (7), and Lohmann (8) observed an increase of the acid-resistant phosphate fraction of tetanized muscle. Lohmann in particular noted a progressive increase of this fraction with increasing length of stimulation. It is more difficult to hydrolyze hexosemonophosphate in  $N$  HCl than most of the other organic phosphorus compounds occurring in muscle and hence the authors quoted above concluded that a hexosephosphate ester accumulates in stimulated muscle. Embden and Jost (9), who devised a direct method for the determination of hexosephosphate in muscle, have not so far published experiments in which pairs of muscles were analyzed for their hexosephosphate content, one unstimulated and the other immediately after a short tetanic contraction, but in referring to the experiments of the authors quoted above they think it probable that an increase in hexosephosphate may occur under certain conditions. Embden and Jost then hasten to point out that such an increase in hexosephosphate during a stimulation, lasting for some time, does not contradict the possibility of a cleavage of this compound during the contraction process itself.

It is now generally believed that during the breakdown of glycogen to lactic acid there occurs an intermediate formation of hexosephosphate, so that whenever lactic acid is formed in muscle, a cleavage of hexosephosphate must take place. According to

Meyerhof (10) the hexosephosphates which have been isolated from muscle, or from cell-free enzyme preparations of muscle acting *in vitro*, are not the true intermediaries of the glycogen  $\rightarrow$  lactic acid transformation but represent stabilization products of a labile form. Embden, however, believes that the mono-ester isolated by him from muscle is the true intermediary or the "lactacidogen" in the strict sense, and hence most of his experiments were devised in an attempt to demonstrate a decrease of this ester during contraction.

Some indication was found in previous experiments that activity leads to an increase in the hexosephosphate content of mammalian muscle. When rats were killed by stunning or decapitation, procedures which lead to intense muscular contractions, the hexosephosphate content was 2 to 4 times higher than in resting muscle (11). Indirect tetanic stimulation of muscle had the same effect. It seemed of interest to determine whether the hexosephosphate accumulating during contraction could be of any significance for lactic acid formation after contraction. Consequently, an attempt was made to strike a balance between disappearance of glycogen and accumulation of hexosephosphate and lactic acid immediately after the contraction and to determine the fate of these constituents some time after contraction.

#### EXPERIMENTAL

All experiments were performed on the gastrocnemius muscle of rats fasted previously for 18 to 20 hours. Shortly before it was desired to stimulate the muscle, amytal was given intraperitoneally. The resting muscle of one side was extirpated for analysis and served as control. The sciatic nerve of the other side was exposed and cut and placed on an electrode connected with the secondary of a Harvard induction coil. A supermaximal stimulation at a rate of 80 per second for 15 seconds was used. The average time elapsing between the end of the stimulation and placing of muscle in trichloroacetic acid or potash solution, as the case may be, was  $30 \pm 5$  seconds. The analytical procedures were as follows: For glycogen the Somogyi<sup>1</sup> modification of the Pflüger method was used, which gave excellent results with a

<sup>1</sup> Personal communication.

considerable saving of time; otherwise the procedure was similar to that described in a previous paper (12). The sugar in the glycogen hydrolysate was determined by the Shaffer-Hartmann method and in some cases also by the Hagedorn-Jensen method with very good agreement and it was ascertained by means of yeast fermentation that non-sugar reducing substances were absent or below 15 mg. per cent. For lactic acid or hexosephosphate determinations the muscle, after remaining for a few minutes in ice-cold 5 per cent trichloroacetic acid solution, was ground very thoroughly with acid-washed sand. In previous experiments (13) in which lactic acid was determined, the muscle was ground in 2 per cent HCl and proteins were precipitated with mercuric chloride. It was found that trichloroacetic acid added in such quantities to zinc lactate solutions, as used in the present determinations in muscle, did not affect the amount of lactate recovered when the Wendel (14) modification of the Friedemann-Cotonio-Shaffer procedure was used. Hence it seemed justified to carry out lactic acid determinations in trichloroacetic acid filtrates of muscle after the usual treatment of the neutralized solution with copper sulfate and lime. No essential changes were introduced in the hexosephosphate method (11). The determination of the reducing power can be carried out by means of the Shaffer-Hartmann reagent containing 1 gm. of KI per liter instead of the ferricyanide reagent, if desired.

*Effect of Stimulation on Hexosephosphate Content*—The experiments recorded in Tables I and II were carried out in the spring of 1931. They show very clearly that a short tetanic stimulation produces a marked increase in the hexosemonophosphate content of muscle. Numerous subsequent experiments at different seasons of the year have never failed to give this result.

Stimulation of the muscle under anaerobic conditions, *i.e.* after cutting off the blood supply as shown in two experiments in Table I, also leads to an increase in hexosephosphate. In these two cases the results of the hydrolysis curve of the trichloroacetic acid filtrate of muscle in *N* HCl may be used to calculate the amount of inorganic P esterified during contraction, because, owing to the ligation of the blood vessels, phosphates cannot escape into the blood stream. In the first case the values for inorganic plus phosphocreatine P, for 7 and 240 minutes of hydroly-

sis and for total acid-soluble P were 98, 137, 160, and 171 mg. per cent in the resting and 89, 125, 150, and 172 mg. per cent in the stimulated muscle. It may be seen that the fraction difficult to hydrolyze in  $N$  HCl (which is indicated by the difference between the 240 minute and total P value) increased from 11 before to 22 mg. per cent after stimulation. The amount of phosphate esterified may also be found by subtracting the 7 minute hydrolysis value of the stimulated from that of the resting muscle; namely,  $137 - 125 = 12$  mg. per cent of P. This happens to be in good agreement with the increase in hexosephosphate P as found by direct analysis; namely, from 9.5 to  $21.9 = 12.4$  mg. per cent of P. In the second case the agreement was not so good; *i.e.*, the phos-

TABLE I

*Indirect Tetanic Stimulation of Rat Gastrocnemius*

Hexosephosphate is expressed in terms of hexose and of P.

All values are given in mg. per 100 gm. of muscle.

Resting			Stimulated			
Hexose	P found	P calculated	Hexose	P found	P calculated	Length of stimulation
						<i>sec.</i>
54	8.4	9.3	95	15.4	16.4	5
49	8.2	8.4	116	20.7	20.0	15
55	7.2	9.5	127	20.1	21.9	15*
56	8.2	9.7	97	18.6	16.7	30*
65	10.4	11.2	117	20.2	20.2	60

\* Blood vessels ligated immediately before stimulation.

phate esterification calculated from the hydrolysis curve was 10 and from the hexosephosphate analysis 7 mg. per cent of P.

A tetanic stimulation of 15 seconds duration caused a decrease in the 7 minute hydrolysis value of only 2 to 3 mg. per cent; *i.e.*, adenosinetriphosphate remained practically unchanged.

It seemed of interest to determine whether the hexosephosphate content of muscle would rise still further when it was already increased at the time of stimulation. Use was made of the observation recorded in a previous paper (11) that epinephrine injections cause an increase in the hexosephosphate content of muscle. The results are shown in Table II; in all cases the

hexosephosphate content showed a further rise after stimulation. In the last two experiments in Table II in which blood vessels were ligated, esterification calculated from the hydrolysis curve amounted to 8 and 15 mg. per cent of P, while the increase observed in hexosephosphate was 9 and 10.7 mg. per cent of P.

*Disappearance of Hexosephosphate after Stimulation*—Experiments in which the rate of disappearance was determined are recorded in Table III. As a control procedure the hexosephosphate contents of the right and left gastrocnemii of the same rat were compared immediately after stimulation and closely agreeing values were obtained; namely, an average of 148 mg. per cent on

TABLE II

*Effect of Stimulation after Epinephrine Injection on Hexosephosphate of Gastrocnemius*

Epinephrine (0.02 mg. per 100 gm. of rat) was injected 30 to 60 minutes previously in order to produce a high initial hexosephosphate content.

All values are given in mg. per 100 gm. of muscle.

Resting			Stimulated			
Hexose	P found	P calculated	Hexose	P found	P calculated	Length of stimulation
						sec.
81	14.5	14.0	118	24.1	20.7	15
93	15.8	16.0	125	24.3	21.6	15
107	16.4	18.4	159	27.0	27.4	15*
116	19.0	20.0	178	29.4	30.7	15*

\* Blood vessels ligated immediately before stimulation.

one and of 147 mg. per cent (in terms of hexose) on the other side. 5 minutes after stimulation the hexosephosphate content had diminished from an average of 137 to 92 mg. per cent and 10 minutes after stimulation it had practically returned to the pre-contraction level, which is 50 to 60 mg. per cent.

There is unfortunately no direct way of proving that the hexosephosphate which disappears in the muscle of the intact animal is converted to lactic acid, because the latter can be disposed of in various ways, either by being carried away by the blood stream, or by oxidation, or by reconversion to glycogen. The assumption is one of analogy and is based on the behavior of hexosephosphate *in vitro* when acted upon by the muscle

enzymes (15). When the blood vessels are ligated immediately after stimulation of the muscle, the hexosephosphate content diminishes markedly in 10 minutes, showing that it can disappear under anaerobic conditions. Here at least there can be little doubt that the end-product is lactic acid.

TABLE III

*Disappearance of Hexosephosphate after Stimulation of Gastrocnemius*

Muscles A and B were stimulated simultaneously for 15 seconds.

All values are given in mg. per 100 gm. of muscle.

Muscle A			Muscle B			Remarks
Hexose	P found	P calculated	Hexose	P found	P calculated	
156	28.6	26.9	138	24.9	23.8	Muscles A and B removed simultaneously
118	20.5	20.3	151	26.1	26.0	
176	31.8	30.3	157	29.9	27.1	
142	21.1	24.5	141	24.2	24.3	
148	25.5	25.5	147	26.3	25.3	
117	20.4	20.2	81	13.2	13.9	Muscle B removed 5 min. after A
134	23.2	23.1	110	17.5	19.0	
131	23.9	22.6	78	13.9	13.5	
164	31.5	28.3	98	18.4	17.0	
137	24.7	23.6	92	15.7	15.8	
124	21.0	21.4	56	9.1	9.6	Muscle B removed 10 min. after A
151	25.9	26.1	53	7.2	9.1	
91	16.3	15.7	43	6.3	7.5	
141	30.0	24.3	71	12.0	12.3	
140	24.0	22.7	77	13.4	13.4	
129	23.4	22.0	60	9.6	10.4	

*Changes in Glycogen and Lactic Acid*—A tetanic stimulation of 15 seconds caused an average decrease of glycogen from 549 to 356 = 193 mg. per 100 gm. of muscle (Table IV). The question is, what percentage of the disappearing glycogen can be accounted for and what are the end-products? In Table III there are seventeen experiments in which the hexosephosphate content was determined immediately after a tetanus of 15 seconds; these

average 139 mg. per cent (in terms of hexose) as compared with a value of 56 mg. per cent before contraction. Hence, the hexose-phosphate content rose 83 mg. and the remainder of the disappearing glycogen, namely  $193 - 83 = 110$  mg., should be

TABLE IV

*Disappearance of Glycogen during Stimulation*

Gastrocnemius muscle was stimulated for 15 seconds in each case.

All values are given in mg. of glucose per 100 gm. of muscle.

Resting	Stimulated
490	333
576	376
477	262
547	381
578	326
627	476
492	326
607	372
549	356

TABLE V

*Accumulation of Lactic Acid during Stimulation*

Gastrocnemius muscle was stimulated for 15 seconds in each case.

All values are given in mg. per 100 gm. of muscle.

Resting	Stimulated
29.3	171
15.7	161
12.9	134
	168
15.2	100
25.2	127
18.8	122
19.5	140

accounted for as lactic acid, provided that there is no other intermediary formed from glycogen during muscular contraction. The average increase in lactic acid of 120.5 mg. found in the experiments in Table V is close to the calculated figure of 110 mg. *It*

may therefore be concluded that the glycogen which disappears from muscle during a short tetanus is accounted for by the hexosephosphate and lactic acid formed. As much as 43 per cent of the glycogen which is broken down during contraction remains in muscle as

TABLE VI

*Recovery of Glycogen after Stimulation*

Gastrocnemius muscle was stimulated for 15 seconds in each case.

All values are given in mg. of glucose per 100 gm. of muscle.

Min. after stimulation			
0		10	20
259	258		
299	298		
339	312		
416	410		
378	395		
338	334		
391		455	
239		312	
213		314	
385		440	
254		304	
227		251	
321		363	
401		436	
232		275	
296		350	
		249	316
		317	360
		315	344
		301	335
		295	339

hexosephosphate and the disappearance of the latter in the next 10 minutes may be regarded as an important source for the lactic acid formation after contraction.

In order to study the recovery of glycogen after contraction, it was necessary to determine whether right and left gastroc-

nemii contained similar amounts of glycogen after both muscles had been stimulated simultaneously with the same current. Such control experiments are recorded in Table VI; on an average the gastrocnemius of one side contained 338 and of the other side 334 mg. per cent of glycogen immediately after a tetanus of 15 seconds. When both muscles were stimulated in the same manner as in these control experiments and one extirpated immediately and the other after 10 minutes, the second muscle contained invariably more glycogen. On an average the glycogen rose 54 mg. in 10 minutes, corresponding to a recovery of 27.9 per cent of the glycogen lost during contraction. A similar comparison between muscles extirpated 10 and 20 minutes after contraction showed that the latter had gained an additional 44 mg. of glycogen, *so that during 20 minutes of rest a muscle may regain 50.8 per cent of its glycogen.* It has occasionally been stated that amytal, the anesthetic under which these experiments were conducted, retards the resynthesis of muscle glycogen. This effect, if it exists under light anesthesia, cannot be very marked because experiments performed without anesthesia on animals with spinal transection gave approximately the same results.

There are two possible sources for the resynthesis of glycogen after contraction; namely, lactic acid and blood sugar. Immediately after contraction the lactic acid concentration in muscle is considerably above that in blood, so that some lactic acid might be expected to diffuse. Another part might be reconverted to glycogen. At the same time, if the present thesis is correct, lactic acid would constantly be formed during the recovery period from the hexosephosphate which disappears. In fact, in 10 minutes 69 mg. of hexosephosphate disappeared, which would be more than enough to account for the glycogen resynthesized during that time. Lactic acid determinations in muscle showed that 10 minutes after contraction the values were still somewhat above normal, while after 20 minutes they had returned to the resting level. Some lactic acid would thus be available for glycogen synthesis even during the period from 10 to 20 minutes. Resynthesis of muscle glycogen at the expense of blood sugar may set in at the beginning of the recovery period or it may start in later; *i.e.*, between 10 and 20 minutes in the present experiments. Certainly, for a full recovery of the glycogen lost during contraction the participation of blood sugar is necessary.

## DISCUSSION

In the present experiments on mammalian muscle a large percentage of the disappearing glycogen had not reached the lactic acid stage when the muscle was analyzed about 30 seconds after the end of the contraction. That part of the disappearing glycogen which was not converted immediately to lactic acid could be accounted for by a corresponding increase in hexosephosphate. A rise in hexosephosphate has also been noted in tetanized frog muscle. During a tetanic contraction glycogen is broken down very rapidly and it is possible that hexosephosphate accumulates because the rate of lactic acid formation does not keep pace with that of phosphorylation, so that there is occasion for the hypothetical labile phosphate ester to be converted into the stable Embden ester.<sup>2</sup> Such a mechanism might serve to retard accumulation of lactic acid in a tetanized muscle. However, if lactic acid formation lags behind, the restitution of phosphocreatine becomes incomplete and hence a tetanized muscle shows a marked decrease of its phosphocreatine content.

A continuation of lactic acid formation during the recovery period would help the muscle to regain its former phosphocreatine content. Such a lactic acid formation, outlasting the contraction process for several minutes, has been observed in frog muscle and seems to be responsible for the delayed recovery heat in the myothermic measurements of Hartree and Hill (16). For obvious reasons it has not been possible to demonstrate an after formation of lactic acid in a mammalian muscle with intact circulation, but the following observation appears to be significant. The hexosephosphate accumulating during contraction was found to disappear again during 5 to 10 minutes of rest and during the same period there was an approximately equal rise in muscle glycogen. It is, of course, possible that the hexosephosphate which disappeared, underwent oxidation and that the muscle glycogen was formed from other sources. In view of the behavior of hexosephosphate when added to a cell-free enzyme extract of muscle, it seems more likely that the hexosephosphate was broken down to lactic acid and that most of this lactic acid was resynthesized to

<sup>2</sup> The stability of the Embden ester is shown by its relatively slow rate of disappearance after contraction.

muscle glycogen. If all the glycogen which disappears during a tetanus were immediately converted to lactic acid, a large part of it would be carried away by the blood stream and would thus be lost to the muscle. With the relatively slow rate of lactic acid formation from hexosephosphate during the recovery period, the resynthesis of muscle glycogen would be able to keep pace, and hence the mechanism here described would make for the preservation of muscle glycogen.

The rate at which hexosephosphate disappears from frog muscle during the recovery period and its relation to the after formation of lactic acid and restitution of glycogen have not as yet been determined. Such experiments will have to be performed before a definite interpretation can be given to the results obtained on mammalian muscle.

#### SUMMARY

1. Indirect tetanic stimulation of the gastrocnemius of the rat for 5 seconds or longer increased the hexosephosphate content of the muscle 2 to 3 times. When the hexosephosphate content was high owing to a preceding injection of epinephrine, electric stimulation caused a further rise. 5 minutes after stimulation the hexosephosphate content had markedly diminished and in 10 minutes it had practically returned to the resting value.

2. The hexosephosphate ester accumulating in muscle as the result of a tetanic stimulation of 15 seconds duration accounted for 43 per cent of the total amount of glycogen which disappeared; the remainder of the disappearing glycogen was accounted for by lactic acid formation. During 20 minutes of rest the muscle regained close to one-half of the glycogen lost during contraction.

3. The possibility of hexosephosphate being a source of lactic acid formation after contraction is discussed.

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# METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

## V. NITROGEN UTILIZATION\*

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The only method of study available for the determination of the progressive nitrogen needs of a woman during a complete normal reproductive cycle is an investigation of her nitrogen balance and of the intermediary metabolism in different stages of pregnancy, lactation, and post lactation.<sup>1</sup> As yet, however, an actual quantitative measure of the nutritive factors essential for the preparation and protection of the maternal organism during child-bearing and child rearing remains undefined. An accumulation of information from long time studies on women is urgently needed to supplement the records now available and thereby furnish facts from which practical dietary applications may be made. When sufficient data from nitrogen balance studies become available, the empirical dietary standards now in use can be replaced.

From a review of the literature on the nitrogen metabolism of women during reproduction it has been found that a preponderance of nitrogen balances have been made just prior to delivery (1-7)

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<sup>1</sup> Data on the intermediary metabolism of the women whose nitrogen balances are herein given will be reported in the future.

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and in the puerperium (1-6, 8, 9). From the 954 daily nitrogen balances available in the literature, 30, 29, 71, 81, 131, 122, 134, and 356 days of balances have been reported for the 3rd to the 10th lunar months respectively. Out of 223 daily balances in the puerperium, 112, 75, 28, and 8 days of balances were made during the 1st, 2nd, 3rd, and 4th weeks respectively. On the other hand, there is a paucity of observations extending throughout gestation and a lack of balance studies in lactation and post lactation. In fact, Hoobler (10) was the only investigator reporting nitrogen balances on women during mature milk secretion prior to the report from this laboratory (11).

The nutritional and physiological requisites of women during pregnancy and lactation have been discussed by several workers (7-21) and for this reason are omitted here. In estimating the maternal demands *in toto* for pregnancy, it is necessary to consider first, the requirements of the fetus and fetal structures including both the placenta and amniotic fluid, and secondly, the maternal preparation for the demands of labor and for future lactation. In the subsequent lactation, the essentials for both the elaboration of milk and the maternal physiological demands of milk secretion must be met. If the maternal needs have not been satisfied during pregnancy and lactation, will there be an immediate replacement of the maternal tissue during post lactation or will there be a lag in this physiological process, thus leaving the mother in a nutritionally unstable state? Furthermore, if a woman enters maternity in a nutritionally unstable condition, what will be the result for her and for her child?

In order to arrive at even an approximation of the total accretion of the fetus and its adnexa it is necessary to resort to computations from a few analyses of fetuses and from a small number of chemical determinations on placentas and amniotic fluid, all of which have been made available through adverse conditions in pregnancy or birth. The analyses of fetuses show an average accumulation of 70 gm. of nitrogen by the end of gestation (22, 23). The weight of the placenta including the fetal blood and cord increases from 33 gm. at the 3rd lunar month to an average of 796 gm. at term (24), its percentage nitrogen content being 2.26 (25). By the use of these figures (24, 25) it may be computed that the amount of nitrogen in the placenta increases from 0.75 gm. at the 3rd to

18.0 gm. at the 10th month. Moreover, from the studies on the quantity of amniotic fluid at various periods in pregnancy (26) and observations on its composition (27), calculations from average figures show that approximately 0.86 gm. of nitrogen has been utilized in the formation of the amniotic fluid by the end of intra-uterine development.

In the preparation of the maternal organism for gestation and lactation, nitrogen is used in the hypertrophy and development of the genital organs, particularly of the uterus and the breasts. Slemons (3) removed a uterus by supravaginal amputation following a Cæsarean section, which upon analysis yielded 38.75 gm. of nitrogen. It may be reckoned that the uterus increases from 30 gm. in weight to about 1 kilo at term. Likewise, Wilson (8) assumed that the breasts increased 0.5 kilo in weight and judged that about 17 gm. of nitrogen were utilized for this purpose. From the estimations available the fetus, placenta, amniotic fluid, uterus, and breasts consume approximately 145 gm. of nitrogen during development *in utero*.

The amount of nitrogen that is stored in excess of the fetus, the uterus, and the breasts has been termed "rest material" by Hoffström (12). He calculated that the entire retention of nitrogen in his subject was 310.05 gm. throughout gestation. It is evident that this quantity of nitrogen is approximately 200 gm. in excess of the combined requirements of the fetus, uterus, and breasts. Similarly, Slemons (3) and Wilson (8) noted large net nitrogen gains to the maternal body at the termination of gestation. In respect to maternal demands Murlin (28) aptly says: "Stated in terms of the different combinations of protein building stones, or 'stereoisomers,' necessary to set up a new human organism, complete in all anatomical details, the requirements for fetal growth are enormous. Can the mother supply all the building materials from her food, or must she perforce supply some structural elements, chemically speaking, from her own body?"

Except for the pioneer work of Hoobler (10) there is a dearth of nitrogen balance data on women during mature milk flow. There are certain estimations, however, that may be made in approximating the nitrogen requirement for milk production. Peters and Van Slyke (29) in generalizing state that the maternal organism can be maintained in nitrogen equilibrium in lactation "if calories enough

to satisfy requirements of both mother and child are given, with somewhat more than enough protein to cover the amount used in the mother's catabolism plus that in the milk." Rose (30) calculated that 0.75 gm. of additional protein for each ounce of milk produced or that 2 gm. of dietary protein for each gm. of protein in breast milk should be consumed in addition to the maintenance intake. It follows then, that the nitrogen requirement increases with an augmented milk flow. This supposition was found true in a quantitative dietary study made in this laboratory on women during 60 weeks of intensive milk flow (31).

The present study records 144 days of nitrogen balances, 49 days of which were made at intervals in pregnancy, 70 in lactation, and 25 in post lactation. These observations contribute additional information concerning the maternal metabolic response to the physiological changes coincident with the development of the fetus, preparation for lactation, lactation itself, and post lactation. In addition, the results from 1177 days of nitrogen balances on women in pregnancy and puerperium have been compiled from the literature to obtain evidence from a large number of cases with which to compare the data secured in this investigation.

#### RESULTS AND DISCUSSION

Metabolic balances have been determined on women<sup>2</sup> at intervals during pregnancy, lactation, and post lactation. The methods used in sampling and analyzing the metabolic materials are omitted for the sake of brevity since they have been described elsewhere in this *Journal* (32, 33). In the beginning of a lactation period our studies were made at the 6th week post partum because under ordinary conditions mature milk flow has been established at that time (34). Complete 24 hour specimens of milk were secured during the metabolic balance observations by a standardized method of manually emptying the breasts at 4 hour intervals.

Two types of balances are reported: first, the *net balance* which is the intake minus the sum of the urine and feces; and secondly, the *maternal balance* which includes on the one hand the demand of the fetus and fetal structures, and on the other hand breast milk. Such data allow for a comparison with those in the literature and at the same time give the nutritional state of the mother

<sup>2</sup> A description of the subjects has been reported elsewhere (11, 32, 33).

TABLE I  
Daily\* Nitrogen Balances during the Reproductive Cycle. Subject VI

Time of study			Length of study	Weight of subject	Calo-riet intake in food	N outgo			N balance				
Period	Wk of period	days				gm	gm	gm	Net		Maternal		
			Urine	Feces	Total				Per kilo	Milk,† fetus,§ and adrena	Total	Per kilo	
3rd lactation													
Dec., 1927	60th	10	63.1	4615	26.50	17.75	2.00	19.75	+6.75	+0.106	3.26†	+3.49	+0.055
44th pregnancy													
Mar., 1928	20th	4	74.7	3650	23.58	18.03	1.87	19.90	+3.68	+0.049	0.17§	+3.51	+0.047
May "	26th	4	80.6	3040	20.18	15.41	2.86	18.27	+1.91	+0.024	0.49	+1.42	+0.018
June "	30th	4	84.2	3280	21.03	16.35	1.87	18.22	+2.81	+0.033	0.24	+2.57	+0.030
July "	34th	4	86.5	3145	19.08	15.10	1.48	16.58	+2.50	+0.029	0.62	+1.88	+0.022
Aug. "	38th	4	86.9	3160	16.23	11.39	1.54	12.93	+3.30	+0.038	0.99	+2.31	+0.026
44th lactation													
Sept., 1928	7th	4	73.1	3780	24.09	14.83	2.01	16.84	+7.25	+0.099	5.93†	+1.32	+0.018
Feb., 1929	27th	4	65.1	4585	27.63	19.18	2.33	21.51	+6.12	+0.094	5.41	+0.71	+0.011
May "	38th	4	64.7	4835	27.91	19.92	2.23	22.15	+5.76	+0.089	5.34	+0.42	+0.006
Oct. "	63rd	4	65.5	4731	27.84	21.90	1.94	23.84	+4.00	+0.061	4.38	-0.38	-0.006

\* Daily balances were secured by dividing the total balance for the period by the number of days in the period.

† Calories were calculated from accepted figures of food composition (35, 36).

† Breast milk, chemically analyzed.

§ The figures for nitrogen in the fetus were obtained by dividing the values given by Fehling (22) for the protein content by 6.25; the daily increment in the nitrogen content of the amniotic fluid was calculated from the figures of Vonnegut (26) and of the placenta from the data of Higuchi (25).

||| Cod liver oil and yeast period. Balances at the 27th and 38th weeks were reported previously (11).

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in so far as it can be calculated. The results are presented chronologically for each subject. In two cases the series of observations were begun in a late lactation and were followed by balances in a complete reproductive cycle; in the third case the observations were made progressively through gestation, lactation, and post lactation.

*Subject VI*—Ten nitrogen balances were carried out on Subject VI and are shown in Table I. The first study occurred at the end of her third long and heavy lactation and was preceded by a period of decreased milk flow. Coincidentally with the voluntary cessation of this lactation period the fourth pregnancy began and five balance studies were made at monthly intervals in the last half of the term. During the succeeding or fourth lactation, four balances were determined under the demands of continuous heavy milk production.

It is noted in Table I that at the close of the third lactation, when the daily nitrogen consumption was 26.5 gm., there was a high maternal nitrogen retention of 3.49 gm. per day. With a nitrogen intake of 23.58 gm. in the 20th week of pregnancy, the net balance was 3.68 gm. This was the highest nitrogen retention for Subject VI during the entire prenatal period, although the fetal accretion alone is inappreciable at this stage of development. As pregnancy advanced, despite a progressive lowering of nitrogen in the voluntary food intake, the net retentions increased at each successive balance study. These observations are in accord with those of other investigators (8, 12, 14) who have found that the maternal organism meets the augmented demands of the fetus and its adnexa by conserving exogenous protein during the late months of gravidity.

In the succeeding or fourth lactation, with a comparatively high nitrogen intake of approximately 27 gm. of nitrogen daily, the maternal nitrogen retentions were 1.32, 0.71, and 0.42 gm. at the 7th, 27th, and 38th weeks respectively, with a loss of 0.38 gm. at the 63rd week. It is significant to note that the loss of nitrogen recorded at the 63rd week of the fourth lactation, when milk production had been maintained at a constantly high level, is in contrast to a storage of nitrogen at a similar time in the third lactation when the milk flow had been allowed to decrease. Likewise, calcium and phosphorus balances were notably negative when milk production remained at a high level (37).

TABLE II  
Daily\* Nitrogen Balances during the Reproductive Cycle Subject VII

Time of study		Length of study	Weight of subject	Calo- ries intake in food	N intake in food	N output			N balance		
Period	Wk of period					Urine	Feces	Total	Total	Per kilo	Maternal
		days	kg		gm	gm	gm	gm	gm	gm	gm
2nd lactation											
Oct, 1927	50th	10	64.5	4762	29.44	19.92	1.72	21.64	+7.80	+0.121	2.06†
3rd pregnancy											
Feb, 1928	14th	4	66.3	3335	18.32	14.59	1.61	16.20	+2.12	+0.032	0.19§
May "	26th	5	72.1	2925	22.24	13.68	2.31	15.99	+6.25	+0.087	0.49
June "	30th	4	73.1	2825	17.75	15.38	1.68	17.06	+0.69	+0.009	0.24
July "	34th	4	75.0	2795	17.01	12.17	1.73	13.90	+3.11	+0.041	0.62
Aug. "	38th	4	75.6	2685	16.07	11.90	1.90	13.80	+2.27	+0.030	0.99
3rd lactation											
Oct, 1928	7th	4	66.7	4430	22.05	15.61	1.98	17.59	+4.46	+0.067	5.30†
Mar, 1929	26th	4	63.1	4430	25.14	17.57	2.68	20.25	+4.89	+0.077	4.02
May "	36th	4	60.8	5330	25.92	17.64	2.82	20.46	+5.46	+0.090	4.01
Nov. "	62nd	3	61.8	4457	22.40	16.44	2.57	19.01	+3.39	+0.055	2.78
Post lactation											
Feb, 1930	11th	3	66.8	3491	15.35	11.28	2.28	13.56	+1.79	+0.027	
Oct 3-5 1930	46th	3	65.9	4481	16.57	11.09	1.94	13.03	+3.54	+0.054	
" 6-9 "	46th	3	65.9	3753	15.25	11.58	1.83	13.41	+1.84	+0.028	
" 10-12 "	47th	3	65.9	3629	15.24	11.53	3.11	14.46	+0.78	+0.012	

\* Daily balances were secured by dividing the total balance for the period by the number of days in the period

† Calories were calculated from accepted figures of food composition (35, 36)

‡ Breast milk, chemically analyzed

§ The figures for nitrogen in the fetus were obtained by dividing the values given by Fehling (22) for the protein content by 6.25; the daily increment in the nitrogen content of the amniotic fluid was calculated from the figures of Vonnegut (26) and of the placenta from the data of Higuchi (25)

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*Subject VII*—Twelve balances were determined on this woman and are given in Table II. The first was carried on at the 50th week of her second lactation and was followed by five studies in the pregnancy which began 1 month after the voluntary cessation of this lactation. The first study in the succeeding or third lactation came at the 7th week post partum and was followed by others at the 26th, 36th, and 62nd weeks, respectively, during uninterrupted heavy milk flow. Additional observations occurred at the 11th and 46th weeks after deliberate discontinuation of lactation in order to study the bodily readjustment following a reproductive cycle. In conformity with the findings on Subject VI at a similar period of lactation, Subject VII with a nitrogen intake of 29.44 gm. stored 5.74 gm. In both cases the quantity of milk secreted had been intentionally diminished from the former high level of milk production for several months preceding the balance study.

Both the net and maternal balances for Subject VII were positive during pregnancy, but they did not increase in magnitude with each successive monthly study as was true with Subject VI. In this case, the retentions and intakes were unrelated except at the 26th week when the greatest net storage of 6.25 gm. coincided with the greatest intake of 22.24 gm. of nitrogen. The mean retention of 2.89 gm. for the five balance studies, however, compares favorably with the average daily storage of 2.28 gm. in the 954 daily nitrogen balances recorded in the literature.

During the third lactation with a daily nitrogen intake of 22.05, 25.14, 25.92, and 22.40 gm. at the 7th, 26th, 36th, and 62nd weeks, the nitrogen maternal balances were  $-0.84$ ,  $+0.87$ ,  $+1.45$ , and  $+0.61$  gm. respectively. 11 weeks after the voluntary termination of lactation, a positive nitrogen balance of 1.79 gm. was observed on a nitrogen intake of 15.35 gm. Likewise, at the 46th week of post lactation, three successive 3 day periods showed retentions of 3.54, 1.84, and 0.78 gm. of nitrogen on intakes of 16.57, 15.25, and 15.24 gm. respectively.

*Subject VIII*—Eleven nitrogen balance studies were conducted on Subject VIII, two of which were in late pregnancy, five in lactation, and four in post lactation (Table III). At the 30th and 34th weeks of gestation with nitrogen intakes of 15.27 and 11.58 gm. the net balances were  $+0.66$  and  $-0.67$  gm. respectively. It may be noted that the consumption of nitrogen and calories was lower than that of either Subject VI or VII.

TABLE III  
Daily\* Nitrogen Balances during the Reproductive Cycle. Subject VIII

Time of study		Length of study	Weight of subject	Calo- riet intake in food	N outgo			N balance			
								Net		Maternal	
					Urine	Feces	Total	Total	Per kilo	Milk,† fetus,‡ and adnexa	Total
Period		Wk. of period	days	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
3rd pregnancy											
May, 1928.....		30th	4	79.2	12.76	1.85	14.61	+0.66	+0.008	0.178	+0.49
June ".....		34th	4	79.8	10.53	1.72	12.25	-0.67	-0.008	0.62	-1.29
3rd lactation											
Aug., 1928.....		7th	4	70.4	15.33	1.93	17.26	+6.49	+0.092	3.31	+3.18
Jan., 1929.....		27th	4	80.8	18.57	2.40	20.97	+0.82	+0.010	3.13	-2.31
May ".....		42nd	4	81.5	19.57	2.17	21.74	+5.73	+0.070	2.84	+2.89
Sept. ".....		63rd	4	81.4	3871	22.24	21.93	+0.31	+0.004	2.44	-2.13
Oct. ".....		65th	3	81.8	3293	15.44	17.18	+5.19	+0.063	1.59	+3.60
Post lactation											
Jan., 1930.....		13th	●4	81.4	2494	12.36	13.90	+0.36	+0.004		
Sept. 22-24, 1930.....		49th	3	80.9	2405	13.67	15.24	+2.03	+0.025		
" 25-27 ".....		49th	3	80.9	2872	12.53	14.15	+3.31	+0.041		
" 28-30 ".....		50th	3	80.9	3190	13.36	15.48	+1.41	+0.017		

\* Daily balances were secured by dividing the total balance for the period by the number of days.

† Calories were calculated from accepted figures of food composition (35, 36).

‡ Breast milk, chemically analyzed.

§ The figures for nitrogen in the fetus were obtained by dividing the values given by Fehling (22) for the protein content by 6.25; the daily increment in the nitrogen content of the amniotic fluid was calculated from the figures of Vonnegut (26) and of the placenta from the data of Higuchi (25).

|| Cod liver oil and yeast period. Balances at the 27th and 42nd weeks were reported previously (11).

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Throughout lactation, Subject VIII demonstrated a markedly variable nitrogen metabolism which seemed unrelated to either the nitrogen intake or its outgo in milk except that at the 65th week, when milk flow had been suppressed approximately one-half, a positive nitrogen balance occurred. In the 7th, 27th, 42nd, 63rd, and 65th weeks of lactation with intakes of 23.75, 21.79, 27.47, 22.24, and 22.38 gm. of nitrogen the maternal balances were +3.18, -2.31, +2.89, -2.31, and +3.60 gm. respectively.

At the 13th week of post lactation with a daily intake of 14.26 gm. of nitrogen there was a nitrogen storage of 0.36 gm. Three successive 3 day studies at the 49th week with intakes of 17.27, 17.46, and 16.89 gm. of nitrogen revealed retentions of 2.03, 3.31, and 1.41 gm. respectively. Such figures, therefore, are comparable to those found for the metabolism of Subject VII at a similar time in post lactation.

These periodic observations on three subjects over a period of 4 years are suggestive but they only indicate the metabolism of the woman at the time of the metabolic balance study. Our women have shown a propensity for nitrogen retention during pregnancy similar to that reported by others (1-8, 12-15). A like ability was shown in lactation, indicating that they were capable of retaining nitrogen sufficiently well to maintain equilibrium notwithstanding their frequent pregnancies followed by long periods of high milk flow. There is a marked variation in the storage of nitrogen in the same individual despite the maintenance of a fairly constant consumption of protein, when consecutive balances are determined. Such evidence indicates that a single balance does not represent a physiological constant of metabolism and consequently it should not be used too freely in generalizations. Nevertheless, attention should be drawn to the fact that, while continuous long time studies are to be desired, an accumulation of data from large numbers of individual balances will yield suggestive information concerning the trend of metabolism during the reproductive cycle.

Accordingly, figures have been assembled from the literature on nitrogen metabolism during pregnancy and puerperium to form a basis for the comparison of the balancedata accumulated in this investigation. The intake and retention values were calculated

in terms of the daily balance and the periods were weighted according to the number of days included in the study. All of these data were collected during short isolated periods varying from 1 to 10 days in length and consequently convey with them the errors of metabolic adjustment to diet as well as many other factors that cause fluctuations. By means of a compilation of 954 daily nitrogen balances it was found that the mean balances were +2.84, -0.15, +1.99, +2.59, +2.18, +2.18, +3.23, and +3.46 gm. for the 3rd to the 10th months respectively. By means of this compilation it will be noted that there is an increase in the average amount of nitrogen stored daily in the

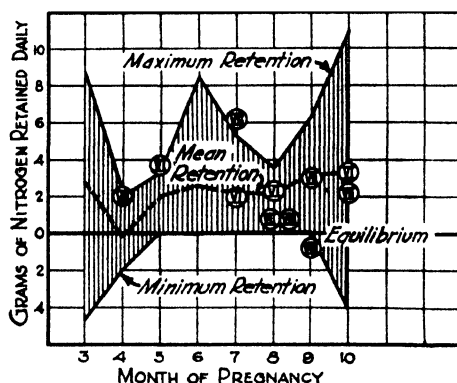


CHART I. Gives the daily nitrogen balances in relation to lunar months of gestation. It shows the maximum, minimum, and mean values of the 954 daily nitrogen balances reported on women in the literature. The results from the isolated nitrogen balance studies on Subjects VI, VII, and VIII are placed in their respective positions on the curve.

maternal organism as pregnancy progresses from the 4th month to term. However, the average daily retention throughout pregnancy is 2.28 gm. Chart I illustrates the results of the nitrogen metabolic studies of Subjects VI, VII, and VIII as compared with those reported in the literature. 48 per cent of the 954 balances fall within the range of 1 to 3 gm. of nitrogen retention daily.

The available data of 223 daily balances for the first 4 weeks of puerperium show that the average nitrogen balances change progressively from -3.12, -0.78, +1.75, and +4.33 gm. during

this time. This evaluation of data on the nitrogen retentions as recorded in the literature was made according to time in the reproductive cycle and irrespective of intake.

Whatever relation the amount of intake may have to the retention of nitrogen during periods of augmented demand, such as in pregnancy and lactation, is important. From the compilation of the data in the literature, it is evident that there are marked variations in the retentions on the same level of intake; some of the low intakes were accompanied by small storages and some of the high intakes by corresponding high retentions.

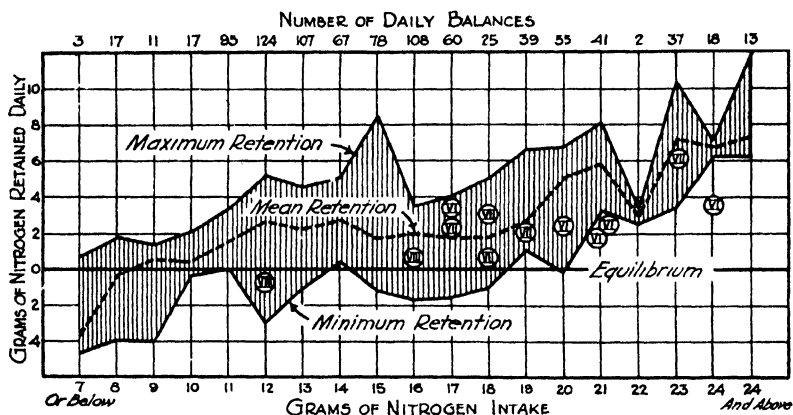


CHART II. Gives the daily nitrogen balances on women in relation to the amount of nitrogen consumed during gestation. It shows the range and the mean values of nitrogen retention of the 917 daily balances reported in the literature. For purposes of comparison, the values for the isolated nitrogen balance studies made on Subjects VI, VII, and VIII are placed in their respective positions on the curve.

A frequency distribution according to quantity ingested and retention shows that 80 per cent of the intake extended from 10 to 18 gm. of nitrogen per day and the accompanying mean retentions fall within the narrow range of 1.5 to 2.7 gm. This constancy of storage is illustrated in Chart II where the nitrogen balances of Subjects VI, VII, and VIII show a similar tendency in response to various degrees of intake. A large loss of nitrogen, however, during the puerperium in spite of the amount consumed is recorded in the literature. This is in accordance with clinical experience.

## SUMMARY

The observations reported in this investigation in combination with the existing data from the literature are a survey of the present knowledge concerning the relation of nitrogen retention in women to the demands of the reproductive cycle. The records indicate that the maternal body retains in pregnancy a considerable excess of nitrogen beyond that required for the fetus and its adnexa. Following parturition, there is a loss which persists over the first 2 weeks or more of the puerperal period. There is evidence that the mother has the ability to maintain nitrogen equilibrium during established lactation and post lactation. In short, the nitrogen metabolic balance studies although of brief duration would indicate that under favorable circumstances the reproductive cycle is a period of nitrogen acquisition for the mother.

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## THE STROPHANTHINS OF STROPHANTHUS EMINII

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In accordance with a plan to investigate as fully as possible the essential chemistry of the strophanthins and other cardiac glucosides, we have attempted to include for comparative purposes, as opportunity presented itself, a study of the digitaloid glucosides and aglucones which occur in the different species of *Strophanthus* plants. Our own and previous investigations have had to do principally with the glucosides of *Strophanthus kombe*, *Strophanthus hispidus*, *Strophanthus gratus*, and *Strophanthus sarmentosus*. And now a further opportunity has been offered to include a study from this standpoint of the seeds of *Strophanthus eminii*. Through the very generous cooperation of the Director of Agriculture of Tanganyika Territory, arrangements were made for the collection by field officers of his department of seeds of this *Strophanthus* species. 20 pounds of the cleaned seeds were procured in September, 1929, in the Shinyanga and Kahama areas of this territory. They were found by us to give uniformly a rose color with sulfuric acid. An earlier sample of such seeds collected in 1925 was stated by the Director to have been identified at the Royal Botanical Gardens, Kew, England, as being those of *Strophanthus eminii*.

As far as previous literature on the glucosides of *Strophanthus eminii* is concerned, the only reference which we have been able to find is the brief statement of Thoms<sup>1</sup> that the strophanthin of *Strophanthus eminii* is different from any strophanthins known at that time.

During the present study, we have found the glucoside mixture to be of complex character. It consists of easily hydrolyzable glucosides and of more stable glucosides. The former, which were

<sup>1</sup> Thoms, H., *Ber. pharm. Ges.*, **14**, 114 (1904).

not isolated in crystalline form, are glucosides of an  $\alpha$ -desoxy sugar because of the typical Keller-Kiliani reaction given by the crude mixture. At first the aglucone obtained on hydrolysis of the labile glucosides gave analytical figures which suggested a formula  $C_{23}H_{34}O_6$ . But on attempting to make certain derivatives, we found it to be a mixture of strophanthidin, the aglucone of *Strophanthus kombe* and *Strophanthus hispidus*, and of periplogenin, which has heretofore been found only as the aglucone of the glucosides of *Periploca græca*.<sup>2</sup> On repeated recrystallization of the mixed aglucones, strophanthidin itself was finally obtained from the mixture. The presence of periplogenin, however, was shown by the isolation of derivatives such as dihydroperiplogenin and *dihydroperiplogenin benzoate*.

The more stable glucosides were isolated as a chloroform-soluble *monoside*,  $C_{30}H_{46}O_9$ , and a *bioside*,  $C_{36}H_{56}O_{11}$ , which was sparingly soluble in chloroform. Both of these glucosides proved to be derivatives of the same aglucone,  $C_{23}H_{34}O_6$ . The latter could not be isolated as such, because of the severe hydrolytic conditions needed for cleavage of the glucosidic linkage. By a special procedure in which methyl alcoholic hydrochloric acid was used, a *trianhydro derivative*,  $C_{23}H_{28}O_8$ , was obtained which, while resembling it closely, proved to be isomeric with *trianhydroperiplogenin* produced by similar treatment of periplogenin itself. Although this would indicate that the previous aglucone is not periplogenin but a closely related isomer, it is still possible that periplogenin in stable glucosidic union could give such an isomeric trianhydro derivative owing to an altered course of dehydration. However, the toxicity of both the monoside and bioside (0.5 mg. failed to kill a 33 gm. frog) was far less than that of periplocymarin. It so happened that the new glucosides were first obtained from seeds which were already practically 3 years old. This fact, together with the low toxicity of these glucosides, gives rise to the suspicion that a certain amount of allomerization, such as had already been shown in the case of *Strophanthus kombe* seeds (allocymarin),<sup>3</sup> may have occurred during this lapse of time and that the monoside and bioside may therefore be glucosides of an alloperiplogenin.

<sup>2</sup> Lehmann, E., *Arch. Pharm.*, **235**, 157 (1897). Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 519 (1928).

<sup>3</sup> Jacobs, W. A., *J. Biol. Chem.*, **88**, 519 (1930).

An attempt will be made to determine this point definitely by an early investigation of fresh seeds which are expected this autumn.

In the above monoside, the aglucone appears to be conjugated with a methyl ether sugar,  $C_7H_{14}O_6$ , which is either identical or isomeric with digitalose, the sugar of *digitalinum verum*. In the bioside there is an additional hexose, possibly glucose, attached to this sugar. Owing to the limited amount of material at our disposal, we have been forced to leave some of these points incomplete for the moment.

#### EXPERIMENTAL

*Isolation of the Glucosides and Aglucones*—2900 gm. of the seeds were finely ground and defatted with petroleum ether. The defatted seeds, which weighed 2200 gm., were thoroughly extracted with 95 per cent alcohol. The alcoholic extract was precipitated with basic lead acetate solution, and the excess lead was removed from the filtrate with hydrogen sulfide. After concentration to a syrup, the residue was taken up in 3 liters of water and the solution was extracted three times with chloroform. The extract, after concentration to about 100 cc. and dilution with petroleum ether, yielded a resinous precipitate which weighed about 15 gm. when dried. This material was then digested for about 15 minutes with boiling water. The aqueous extract was filtered hot from the water-insoluble resins. On cooling, about 0.5 gm. of the crude monoside crystallized out. The resinous residue was digested again with the mother liquor from this crystalline material. Concentration of the filtered solution gave 0.2 gm. more of crude monoside.

The aqueous solution, which remained after the above chloroform extraction, was freed from chloroform and its reaction was made barely acid to Congo red with hydrochloric acid. Then sufficient hydrochloric acid was added to make its effective concentration 0.15 per cent. The solution was warmed to 75° and held at this temperature for 1 hour. After about 15 minutes, the aglucone mixture of strophanthidin and periplogenin began to separate, sometimes in the form of mixed crystals and sometimes as a thick oil which crystallized on standing. When the hydrolysis was complete, the solution was cooled and allowed to stand for several weeks in order to complete crystallization. The precipi-

tate was then collected with water. About 20 gm. of crude mixed genins were thus obtained. The mother liquor was extracted three times with chloroform. The dried and concentrated chloroform extract, on dilution with petroleum ether, yielded about 3 gm. of an amorphous material which, on recrystallization from ethyl alcohol, proved also to be a mixture of strophanthidin and periplogenin.

The aqueous mother liquor from the genin mixture was neutralized to Congo red with sodium acetate, and was then concentrated under diminished pressure to a volume of about 400 cc. Octyl alcohol was used to check persistent foaming. The concentrated solution was salted out with ammonium sulfate. The supernatant liquid was decanted from the gummy precipitate which was kneaded with a rod in order to free it from as much included mother liquor as possible. The mass was then dissolved in 1 liter of absolute alcohol. The filtrate from undissolved ammonium sulfate was concentrated under diminished pressure to a thick syrup. This was in turn taken up in about 300 cc. of water. A saturated solution of ammonium sulfate was then cautiously added to the solution until the curdy precipitate first formed barely redissolved. The solution was then seeded. After a week or so, the bioside very slowly crystallized. The suspension was allowed to stand at least a week more to insure complete crystallization. The crystals were best collected by centrifuging. The crude product was suspended in a small quantity of water and again centrifuged. After mixing again with water, it was readily collected on a filter. The crude bioside weighed 2.0 gm.

The mother liquor remaining after the above isolation of the active constituents was still quite rich in glucosides, but all attempts to isolate them in crystalline form have until now been unsuccessful.

*The Monoside,  $C_{30}H_{46}O_9$* —The above monoside could be recrystallized only with difficulty, its behavior suggesting the presence of more than one component. After four recrystallizations from dilute ethyl alcohol, the substance was obtained as rosettes of fine needles which melted to a frothy mass at 174–180°, after preliminary sintering. It gave a positive Legal reaction. The Keller-Kiliani test for  $\alpha$ -desose was practically negative. In concentrated sulfuric acid, the substance gave a yellow-brown

solution which passed through a yellow-green to a dull blue-green.

		$[\alpha]_D^{25} = +22^\circ$ ( $c = 0.995$ in 95 per cent alcohol)	
4.045 mg. substance:	3.070 mg. $H_2O$ ,	9.725 mg. $CO_2$	
4.034 " "	: 2.990 " "	9.770 " "	
3.775 " "	: 1.210 " AgI		
$C_{23}H_{34}O_6$ . Calculated.		C 65.41, H 8.42, $OCH_3$ 5.63	
Found.		" 65.59, " 8.49	
		" 66.07, " 8.29	
			" 6.03

The analysis of this glucoside shows it to be a derivative of an aglucone,  $C_{23}H_{34}O_6$ , and a methyl ether desoxy sugar,  $C_7H_{14}O_6$ . The latter is apparently not an  $\alpha$ -desoxy sugar, because of its resistance to the hydrolyzing action of mineral acids. Only a fraction of the glucoside was cleaved by a half hour's treatment with 1 per cent hydrochloric acid in 40 per cent alcohol at  $73^\circ$ . 5 per cent hydrochloric acid in 50 per cent ethyl alcohol hydrolyzed the glucoside fairly rapidly in a half hour's time, but the reaction product was not homogeneous and had poor physical properties. When treated by the following method, the sugar was cleaved and simultaneously 3 molecules of water were split out, with the formation of a trianhydroaglucone.

*The Trianhydroaglucone,  $C_{23}H_{28}O_2$ , from the Monoside*—0.3 gm. of the monoside was dissolved in 2 cc. of a 5 per cent solution of hydrochloric acid in absolute methyl alcohol. The solution was heated in a sealed tube at  $100^\circ$  for 30 minutes. On cooling, 8 mg. of a crystalline substance separated, which melted at  $135$ – $141^\circ$ . After repeated recrystallization from alcohol, it formed lustrous platelets which melted at  $154$ – $156^\circ$ . The melting point was not depressed when the sample was mixed with a similar substance obtained from the bioside.

The trianhydro derivative dissolves in concentrated sulfuric acid with an orange-brown color that turns through a dull red-purple to a dull violet-blue. It gives a positive nitroprusside reaction.

1.977 mg. substance:	1.540 mg. $H_2O$ ,	5.945 mg. $CO_2$
$C_{27}H_{28}O_2$ . Calculated.		C 82.09, H 8.39
Found.		" 82.01, " 8.69

*The Bioside, C<sub>36</sub>H<sub>56</sub>O<sub>14</sub>*—The crude bioside was recrystallized first by dilution of its alcoholic solution and finally from hot water. It formed large, irregular leaflets which melted not sharply at 195–200°, after preliminary sintering. The Legal test was positive. The Keller-Kiliani test was practically negative. The substance dissolved in concentrated sulfuric acid to give a yellow-brown solution, a little darker than that of the monoside, passing in a few hours time through a dull purple to a purple-black.

$[\alpha]_D^{25} = +8^\circ$ ( $c = 0.950$ in 95 per cent alcohol)			
3.182 mg. substance:	2.725 mg. H <sub>2</sub> O,	8.420 mg. CO <sub>2</sub>	
4.246 “ “ :	3.000 “ “	9.390 “ “	
4.548 “ “ :	1.530 “ AgI		
C <sub>36</sub> H <sub>56</sub> O <sub>14</sub> . Calculated. C 60.64, H 7.92, OCH <sub>3</sub> 4.35			
Found. “ 60.24, “ 8.00			
“ 60.31, “ 7.91			
			“ 4.44

This bioside, from the analysis, is a derivative of a methyl ether desoxy bioside, C<sub>13</sub>H<sub>24</sub>O<sub>10</sub>, and an aglucone, C<sub>23</sub>H<sub>34</sub>O<sub>5</sub>. As far as can be determined with the small quantity of material available, this aglucone is identical with that contained in the monoside. As in the case of the latter, only a trianhydrogenin could be obtained, owing to the resistance of the bioside to hydrolysis. A satisfactory cleavage was obtained by the use of the method successfully employed with the monoside, as follows:

*The Trianhydroaglucone from the Bioside*—0.5 gm. of the bioside was heated in 3 cc. of 5 per cent absolute methyl alcoholic hydrogen chloride at 100° for 30 minutes. 40 mg. of the trianhydro derivative crystallized out. Recrystallization from ethyl alcohol yielded irregular platelets which melted at 154–156°, and agreed in all properties with the substance obtained from the monoside.

$[\alpha]_D^{27} = -84.6^\circ$ ( $c = 1.015$ in pyridine)			
4.290 mg. substance:	3.248 mg. H <sub>2</sub> O,	12.882 mg. CO <sub>2</sub>	
C <sub>23</sub> H <sub>28</sub> O <sub>2</sub> . Calculated. C 82.09, H 8.39			
Found. “ 81.93, “ 8.47			

This substance is definitely different from trianhydroperiplogenin, which will be described in a subsequent communication.

*The Aglucone Fraction*—This consisted roughly of equal amounts of strophanthidin and periplogenin. They appeared to form

mixed crystals, the fractionation of which proved to be difficult. 2 gm. of the crude mixture yielded, after seven recrystallizations from 95 per cent alcohol, 40 mg. of a substance which melted at 174–178° and appeared as the characteristic rhombs of strophanthidin.

$$[\alpha]_D^{25} = +35.6^\circ \text{ (} c = 1.005 \text{ in 95 per cent ethyl alcohol)}$$

3.897 mg. substance: 2.840 mg. H<sub>2</sub>O, 9.620 mg. CO<sub>2</sub>

C<sub>23</sub>H<sub>32</sub>O<sub>6</sub> · 0.5H<sub>2</sub>O. Calculated. C 66.79, H 8.05

Found. " 67.33, " 8.15

The presence of strophanthidin in the crude mixture was directly substantiated by the production of the methylal of dianhydrostrophanthidin, as follows:

A solution of 0.2 gm. of mixed aglucones in 2 cc. of a 5 per cent solution of hydrogen chloride in absolute methyl alcohol was heated in a sealed tube at 100° for 15 minutes. On cooling, the crude product crystallized spontaneously. After two recrystallizations from methyl alcohol, 38 mg. of the methylal, melting at 240–244°, were obtained. An additional recrystallization sharpened the melting point to 242–244°, and the substance gave no depression when mixed with an authentic sample of the methylal of dianhydrostrophanthidin.<sup>4</sup>

$$[\alpha]_D^{27} = -124.7^\circ \text{ (} c = 1.000 \text{ in chloroform)}$$

4.132 mg. substance: 2.905 mg. H<sub>2</sub>O, 11.400 mg. CO<sub>2</sub>

3.920 " " : 2.475 " AgI

C<sub>24</sub>H<sub>30</sub>O<sub>4</sub>. Calculated. C 75.35, H 7.91, OCH<sub>3</sub> 8.12

Found. " 75.23, " 7.85

" 8.34

*Hydrogenation of the Mixed Aglucones*—A solution of 0.4 gm. of the mixed aglucones in 40 cc. of ethyl alcohol was hydrogenated in the presence of 0.2 gm. of the platinum oxide catalyst of Adams and Shriner. After reduction of the catalyst, approximately 1 mol of hydrogen was absorbed in about 40 minutes. Absorption continued after this, but too slowly to make the measurement reliable. At the end of 24 hours, the solution was filtered from the catalyst and concentrated to a syrup. When treated with ether, a portion of the product crystallized. This fraction, after repeated

<sup>4</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **59**, 725 (1924).

recrystallization from boiling methyl alcohol, melted constantly at 170–173°. A mixed melting point with a sample of dihydrostrophanthidol showed no depression. The melting point of dihydrostrophanthidol, when recrystallized from dilute methyl alcohol, has been reported in a previous communication<sup>5</sup> as 160–163°. It has been found more recently that after recrystallization from a small volume of methyl alcohol it melts at 172–175°. Shortly after, it resolidifies and melts again at about 195°.

In all other properties, the new substance proved to be identical with dihydrostrophanthidol.  $[\alpha]_D^{29} = +35.0^\circ$  ( $c = 1.030$  in 95 per cent ethyl alcohol).

An authentic sample of dihydrostrophanthidol, recrystallized from methyl alcohol, showed a comparable rotation.

$[\alpha]_D^{27} = +31.8^\circ$  ( $c = 0.980$  in 95 per cent ethyl alcohol)

4.577 mg. substance: 3.620 mg. H<sub>2</sub>O, 11.420 mg. CO<sub>2</sub>

C<sub>22</sub>H<sub>16</sub>O<sub>6</sub>. Calculated. C 67.60, H 8.89

Found. " 68.07, " 8.85

That portion of the crude hydrogenation product which remained soluble in ether was freed from solvent and dissolved in cold methyl alcohol. A small amount of dihydrostrophanthidol, insoluble under these conditions, was filtered off. The filtrate was brought to crystallization by the cautious addition of water. The crude product weighed 180 mg. After several recrystallizations, a substance which melted at 201–203° was obtained. This substance proved to be dihydroperiplogenin.<sup>6</sup> A mixed melting point with dihydroperiplogenin showed no depression. In concentrated sulfuric acid, the substance gave a clear light orange color finally changing to a bright blue identical with that given by an authentic specimen of dihydroperiplogenin. The identity of the substance was confirmed by other properties.  $[\alpha]_D^{27} = +24.8^\circ$  ( $c = 1.01$  in 95 per cent alcohol).

Authentic dihydroperiplogenin gave a rotation of  $[\alpha]_D^{26} = +25^\circ$  ( $c = 1.000$  in 95 per cent alcohol).

4.390 mg. substance: 3.690 mg. H<sub>2</sub>O, 11.330 mg. CO<sub>2</sub>

C<sub>23</sub>H<sub>36</sub>O<sub>6</sub>. Calculated. C 70.35, H 9.25

Found. " 70.39, " 9.40

<sup>5</sup> Jacobs, W. A., *J. Biol. Chem.*, **88**, 528 (1930).

<sup>6</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 528 (1928).

In the course of checking the identity of the ether-soluble hydrogenation product, its benzoyl derivative was also prepared. Benzoylation was accomplished by the usual procedure with benzoyl chloride in pyridine solution. A monobenzoate was formed, which after recrystallization from methyl alcohol, melted at 214–216°.

$$[\alpha]_D^{25} = +46^{\circ} (c = 1.000 \text{ in pyridine})$$

4.210 mg. substance: 3.060 mg. H<sub>2</sub>O, 11.237 mg. CO<sub>2</sub>

C<sub>30</sub>H<sub>40</sub>O<sub>6</sub>. Calculated. C 72.54, H 8.12

Found. " 72.79, " 8.13

Benzoyldihydroperiplogenin similarly prepared from authentic dihydroperiplogenin also melted at 214–216°.

$$[\alpha]_D^{25} = +47.1^{\circ} (c = 1.015 \text{ in pyridine})$$

4.387 mg. substance: 3.230 mg. H<sub>2</sub>O, 11.665 mg. CO<sub>2</sub>

C<sub>30</sub>H<sub>40</sub>O<sub>6</sub>. Calculated. C 72.54, H 8.12

Found. " 72.53, " 8.24



## **$\beta$ -AMINO-*n*-VALERIC ACID**

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Only two  $\beta$ -aminovaleric acids appear to have been described; namely, the derivative of isovaleric acid first obtained by Heintz (1) by the oxidation of acetaminine and subsequently synthesized by Bredt (2) and by Slimmer (3) and the  $\beta$ -amino derivative of trimethylacetic acid prepared by Kohn and Schmidt (4). The following communication describes the preparation of  $\beta$ -amino-*n*-valeric acid. The acid was required for purposes of comparison with a compound, precipitable by phosphotungstic acid and forming a crystalline reineckate obtained in the course of other work carried out in collaboration with the Department of Medicine of Columbia University. It may be noted here that with the exception of isoserine all  $\beta$ -amino acids so far available (the  $\beta$ -amino derivatives of propionic, *n*-butyric, valeric, and caproic acids, and phenylpropionic acid) have been found to yield crystalline relatively sparingly soluble reineckates, thus differentiating them sharply from the corresponding  $\alpha$ -amino acids. The new amino acid was obtained by the action of ammonia under pressure on propylideneacetic acid under conditions resembling those used by Stadnikoff (5) and by Fischer and Scheibler (6) for the preparation of  $\beta$ -aminobutyric acid. Propylideneacetic acid ( $\alpha,\beta$ -pentenic acid) was prepared by the action of quinoline on the ethyl ester of  $\alpha$ -bromo-*n*-valeric acid under the conditions described by Rupe, Ronus, and Lotz (7). The free acid (20 gm.) was neutralized with ammonia and the solution (75 cc.) was then saturated with ammonia gas at about 5°. The mixture was then heated in a steel tube, closed with screw-cap and leaden washer, for 24 hours at a temperature maintained between 125–135° by means of a hot oil bath. The reaction mixture was diluted with water, boiled to remove as much ammonia as possible, and filtered to remove traces

of iron hydroxides. The solution was then evaporated to a syrup under diminished pressure and the residue converted into the ethyl ester hydrochloride by repeated treatment with alcohol and hydrochloric acid gas in the usual fashion. The ester hydrochloride was decomposed with ammonia gas, in the presence of ether (100 cc.), and the ammonium chloride removed by filtration. After drying with sodium sulfate the ether was evaporated off at a low temperature and the residue fractionated under reduced pressure. In addition to a first fraction containing much alcohol and some ester, a large fraction (19 gm.) boiling steadily between 82–84° under 12 mm. pressure was obtained, while only 3 gm. of higher boiling residue were left behind. Ethyl- $\beta$ -amino-*n*-valerate is a pleasant smelling mobile liquid with a much less "basic" suggestion in its odor than similar  $\alpha$ -amino acid esters. It is readily soluble in water and was hydrolyzed by boiling with 10 parts of water under a reflux condenser for 4 hours, after which the alkaline reaction had disappeared. On concentrating to small bulk a clear syrup was obtained, which began to crystallize in a few days and after standing in a desiccator finally set to a solid mass. The recrystallization of the acid is not particularly easy but may be accomplished by dissolving in warm absolute alcohol and then adding dry ether, when rosettes of fine needles separate out in the cold. The crystals are extremely soluble in water though not deliquescent when pure and dissolve fairly readily in warm alcohol but are insoluble in ether. Like many  $\beta$ -amino acids the melting point is not very sharp, as decomposition begins between 160–165° but a clear melt is not obtained below 185°. The substance was dried *in vacuo* over phosphorus pentoxide for analysis.

*Analysis*

$C_6H_{11}O_2N$ .	Calculated.	C 51.2, H 9.4, N 11.9
	Found.	" 51.1, " 9.6, " 11.7

Close to the theoretical amount of nitrogen (11.7 per cent) is obtained on treatment with nitrous acid for 5 minutes in a Van Slyke apparatus, but the carboxyl titration in the presence of formaldehyde in aqueous solution gives results only approximately half the theoretical value, while even on addition of alcohol the results are still slightly low.

**Copper Salt**—On boiling a 5 per cent aqueous solution of the amino acid for half an hour with a large excess of freshly precipitated copper hydroxide, filtering, and concentrating to small bulk, the copper salt separated in the form of clear blue flat shining needles which are readily soluble in cold and hot water. The air-dried crystals contain about 1 molecule of water of crystallization (calculated 10.8, found 11.0 to 13.1) which is lost at  $90^{\circ}$ , the salt assuming a violet tinge. On ignition, the vapors, as expected, give no pyrrole reaction.

*Analysis*

$(C_6H_{10}NO_2)_2Cu$ .	Calculated.	Cu 21.5, N 9.47
	Found.	" 21.0, " 9.37

**Phosphotungstate**—The amino acid is readily precipitated by phosphotungstic acid as a heavy white crystalline compound which was not analyzed. In some early experiments the phosphotungstate was used as a means of isolating the amino acid, after removal of ammonia, from the crude material prepared by heating propylideneacetic acid and ammonia. The yield was somewhat less than that obtained by the ester method and the product crystallized less easily.

**Reineckate**—On adding ammonium reineckate (4 parts) dissolved in warm water to a 5 per cent solution of the amino acid (1 part) and then making just acid to Congo red with dilute sulfuric acid, the reineckate separates out slowly as a crystalline precipitate. It is readily recrystallized from 20 per cent methyl alcohol separating as garnet-red shining plates of considerable size. On drying *in vacuo* at ordinary temperature only a trace of water is retained by the crystals which darken at  $156^{\circ}$  and melt indefinitely between  $171$ – $175^{\circ}$  to a black mass. It is sparingly soluble in water, and readily soluble in dilute alcohol.

*Analysis*

$C_6H_{11}NO_2 \cdot C_4H_7N_6S_4Cr$ .	Calculated.	Cr 12.0, N 22.5
	Found.	" 12.2, " 22.4

**$\beta$ -Uramido-*n*-Valeric Acid**—A 10 per cent solution of the amino acid (1 part) was warmed on the water bath for an hour with potassium cyanate (2 parts). On making just acid to Congo red with dilute sulfuric acid, no separation occurred. The uramido

acid is extremely soluble in water but may be slowly extracted with ether in a continuous extractor. About 50 per cent was extracted in the course of 10 hours and the clear syrup readily crystallized in the desiccator. It is extremely soluble in water and alcohol and sparingly soluble in benzene. It is best crystallized by dissolving in a minimum of warm alcohol and then adding benzene. It crystallizes in needles melting sharply at 147–148° without effervescence.

*Analysis*

$C_6H_{12}O_3N_2$ . Calculated, N 17.5; found, N 17.7

*4-Ethylhydrouracil*—The preceding compound on short boiling with 15 per cent hydrochloric acid is readily converted into its anhydride, 4-ethylhydrouracil, which is sparingly soluble in cold water and separates out as well formed thick prismatic needles. The same compound is more readily obtained without previous isolation of the uramido acid by simply evaporating the reaction mixture obtained from the amino acid and potassium cyanate with excess of hydrochloric acid. It is readily recrystallized from boiling water and melts at 191–192° without apparent decomposition.

*Analysis*

$C_6H_{10}O_2N_2$ . Calculated. C 50.7, H 7.0, N 19.7  
Found. " 50.5, " 6.8, " 19.6

*1-Phenyl-4-Ethylhydrouracil*—The amino acid (1 gm.) reacts readily with phenyl isocyanate (1.2 gm.) in the presence of dilute sodium hydroxide (10 cc., 2.5 N). On acidifying, an oily precipitate which quickly solidifies is obtained, but it has unattractive physical properties. It was therefore converted into the anhydride by evaporation on the steam bath with excess of hydrochloric acid (1:1). The product may be crystallized from hot water but alcohol is decidedly preferable. It is very readily soluble in hot alcohol but only moderately soluble in cold alcohol, separating in thin silky needles melting at 192°. It is moderately soluble in cold ether.

*Analysis*

$C_{12}H_{14}O_2N_2$ . Calculated. C 66.0, H 6.42, N 12.8  
Found. " 65.9, " 6.35, " 12.7

I am indebted to Mr. William Saschek for some of the analyses made with the generous cooperation of Professor H. T. Clarke.

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## A METHOD FOR THE QUANTITATIVE ESTIMATION OF INDOXYL COMPOUNDS IN URINE

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In 1913 Jolles (1) introduced alcoholic thymol as a reagent for the detection of indican in urine, and later, based upon its use, developed a colorimetric method (2) for the estimation of indican in both urine and blood. His method for urine consisted in adding to 10 cc. of urine filtrate, after clarification with lead acetate, 1 cc. of 5 per cent alcoholic thymol, and 10 cc. of concentrated hydrochloric acid containing ferric chloride. This mixture was allowed to stand for 15 minutes, after which it was thoroughly shaken with 4 cc. of chloroform. The chloroform extracted a purple pigment (indoxyl-thymol compound) whose color intensity was alleged to be proportional to the amount of indican present. Jolles claimed to detect by this method 0.0032 mg. of indican in 10 cc. of urine. For quantitative measurements by the usual comparison colorimetry, pure indican was necessary for the production of pigment intensities of known indican value.

Repetition and manipulation of this procedure with both urine and indican solution disclosed that Jolles' method fails to be a satisfactory quantitative method, that the use of many times the amount of acid advised produces pigments of greater color intensity per unit of indican, and that the addition of trichloroacetic acid to the acid reagent induces a red value in the purple hue which increases in direct proportion to the increase of indican in the reaction mixture.

It was possible to adjust the amount of trichloroacetic acid used to form readily within the reaction mixture ethyl trichloroacetate which served as an organic solvent for the indoxyl-thymol condensate produced. The procedure indicated below makes use of the findings just mentioned and is a quantitative method for indoxyl at least  $2\frac{1}{2}$  times as sensitive as Jolles' reaction. For

quantitative measurements we have adapted our technique to the comparison colorimeter by the use of a color filter and an appropriate colored solution as a standard.

In seeking to establish the specificity of this reaction for indoxyl under test conditions we investigated three sources for substances that might react after the manner of indoxyl compounds: (1) other normal urinary constituents which might, when present in unusually high concentration, be reactive, (2) pathological or rare physiological urinary substances, and (3) substances always foreign to urine.

In the first category more than a dozen different substances were tested, first in 1 per cent water solution and then after addition to urine in similar concentration. In no case did any of these substances produce a color reaction under test conditions or on addition to urine quantitatively influence the reaction with the indoxyl contained therein. Removal of urinary pigment by clarification with lead acetate before carrying out the indoxyl determination in no way affected the accuracy of the reaction, proving this step unnecessary under the conditions of our test.

Of rare or pathological substances that might be present in urine we found that glucose, formaldehyde, and tryptophane produced condensation pigments of a carmine color. However, with tryptophane several mg. were necessary to produce the faintest of colors. Added glucose to urine up to 3 per cent or added formalin up to 0.1 per cent concentration effected no change in the indoxyl measurement. Apparently, reactive substances other than indoxyl could be rendered non-interfering by establishing test conditions that brought into the reaction immeasurably small amounts of the interfering substances.

Among the many substances foreign to urine several homocyclic and heterocyclic compounds were tested. In 1 per cent concentration they were all non-reactive. Since toluene is advised as a urinary preservative, it was established that the indoxyl measurements on urine before and after a thorough shaking with toluene were identical. Determinations performed in duplicate on over 500 different samples of urine from persons well and diseased have always produced a pigment condensate of a red-blue hue, comparable to, though not necessarily identical with the one occurring in pure indican solution.

*Determination of Indoxyl Compounds*

*Reagent A*—1 per cent potassium persulfate in distilled water.

*Reagent B*—1 per cent thymol in 95 per cent ethyl alcohol.

*Reagent C*—Acid reagent, made by adding to each 100 gm. of trichloroacetic acid 200 cc. of water and 200 cc. of concentrated hydrochloric acid.

*Reagent D*—Glacial acetic acid.

*Procedure*—5 cc. of urine<sup>1</sup> are placed in a volumetric flask and diluted to 100 cc. with distilled water. To 2 cc. of this dilution in a 15 cc. centrifuge tube (graduated in 0.1 cc.) are added 5 drops of Reagent A, 0.5 cc. of Reagent B, and 5 cc. of Reagent C. Before the addition of the acid reagent, the mixture is shaken for a second or two. The tube is immediately placed in a boiling water bath and kept at that temperature for approximately 5 minutes. Before placing the tube in the water bath, the mixture is slightly cloudy; it quickly clears at the temperature of the water bath, soon followed by the formation of a second and more intense cloud. This is due to the formation of ethyl trichloroacetate which soon begins to settle to the bottom of the tube, carrying down with it a purple pigment (the indoxyl-thymol compound). The tube after removal from the water bath is allowed to stand until it cools (about 15 minutes), when all<sup>2</sup> of the pigment-laden acetate has settled to the bottom of the tube, giving an acetate volume of approximately 0.5 cc. The supernatant watery acid layer is then carefully removed with a pipette, leaving about 0.3 cc. of watery layer above the colored acetate. This mixture (approximately 0.8 cc.) of acetate and acid water is diluted to exactly 2 cc. with glacial acetic acid, delivered from a burette. The mixture is stirred with a glass rod until it clears, giving a perfectly transparent, colored solution. This transparency is always achieved if, in pipetting, care is taken never to leave more than 0.3 cc. of watery layer as overlying residue. The colored solution is now ready to be read.

The reading is done in the ordinary comparison colorimeter,

<sup>1</sup> Urine must not be preserved with formaldehyde; when a preservative is required, toluene may be used.

<sup>2</sup> This is not strictly correct; the last traces must be thrown down by centrifugation.

with micro cups and appropriate plungers. In the eyepiece of the instrument is placed a green filter (Wrattan, No. 74). For a standard solution we use a 1.5 per cent solution of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  (Merck, C. P.) whose indican equivalent is derived from Table II.

TABLE I

*Data for Estimation of Indican Equivalent of Light Absorption Value of Ethyl Trichloroacetate-Acid Mixture*

0.75 per cent  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  was used. The colorimeter cup containing the tested material (last column) was set at 30 mm.

Colorimeter reading			Indican used
mm.	mm.	mm.	mg.
20.0	20.1	20.1	0.002
20.3	20.4	20.3	0.002
20.4	20.5	20.6	0.002
20.1	20.0	20.2	0.002
20.1	20.0	20.2	0.002
27.2	27.3	27.2	0.003
27.4	27.7	27.6	0.003
26.9	27.0	26.9	0.003
27.5	27.6	27.7	0.003
27.2	27.4	27.6	0.003
6.0	6.1	6.2	Blank
6.2	6.3	6.2	"
5.8	5.9	5.8	"
7.0	7.1	7.0	"
5.4	5.5	5.4	"
6.4	6.5	6.4	"

(A)	Average reading	for 0.002 mg. indican	= 20.2 mm.
(B)	"	" 0.003 "	= 27.3 "
(B - A)	" equivalent	" 0.001 "	= 7.1 "
A - 2 (B - A)	"	" blank	= 6.0 "
(C)	" reading	" "	= 6.2 "
	" for above values of blank		= 6.1 "

$$\text{Indican equivalent blank, } \frac{6.1}{7.1} \times 0.001 \text{ mg.} = 0.0009$$

(0.00086)

In a method such as this, we are called upon to compare two green fields, the intensities of which are influenced by the light absorption coefficient of the colored solution interposed between the source of light and the eye. Kennedy (3) has elucidated the

theory of this type of colorimetry; its applicability to the quantitative determination of indican will be apparent from what follows.

It was readily demonstrated that cobalt sulfate solution served perfectly as a color standard for determining indican, provided a correction was estimated and introduced to compensate for the difference in light absorption values between water, the solvent

TABLE II

*Standardization of Cobalt Sulfate Solution As Determined by Use of Different Concentrations of Both Indican and Cobalt Sulfate*

No. of determinations	Indican employed	CoSO <sub>4</sub> used	Factor for concentration of CoSO <sub>4</sub> used*	Factor equivalent per 1.5 per cent CoSO <sub>4</sub>
	mg.	per cent		
3	0.002	0.75	0.0042	0.0084
3	0.003	0.75	0.0042	0.0084
3	0.004	0.75	0.0045	0.0090
1	0.002	1.5	0.0084	0.0084
2	0.004	1.5	0.0086	0.0086
2	0.006	1.5	0.0087	0.0087
2	0.008	1.5	0.0082	0.0082
2	0.010	1.5	0.0086	0.0086
2	0.012	1.5	0.0089	0.0089
2	0.014	1.5	0.0087	0.0087
1	0.008	2.5	0.0146	0.0088
1	0.010	2.5	0.0145	0.0087
1	0.012	2.5	0.0135	0.0081
1	0.014	2.5	0.0132	0.0080
1	0.008	5.0	0.0292	0.0087
1	0.012	5.0	0.0288	0.0086
1	0.016	5.0	0.0295	0.0088
1	0.020	5.0	0.0287	0.0086
Average.....				0.0086

\* Factor =  $\frac{(\text{mg. indican used} + 0.0009) \times \text{indican reading (mm.)}}{\text{CoSO}_4 \text{ reading (mm.)}}$ . When more than one determination was made, the average figure is given.

for cobalt sulfate, and ethyl trichloroacetate, the solvent for the indoxyl-thymol pigment. In Table I are given data which demonstrate the existence of a significant light absorption value for the colorless acetate-acid mixture and from which the indican equivalent of this mixture may be calculated as well. We could

have introduced our correction as mm. to be subtracted from the readings, but this would have necessitated either always maintaining the unknown solution at 30 mm. or introducing a variable

TABLE III  
*Indoxyl (As Indican) Determination on Addition of Indican to Urine*

Urine No.	Indican found per 0.1 cc. urine	Indican added per 0.1 cc. urine	Total indican determined	Per cent of calculated total
	mg.	mg.	mg.	
1-A	0.0038	0.0113	0.0168	112
1-B	0.0038	0.0113	0.0173	
2-A	0.0199	0.0113	0.0311	104
2-B	0.0202	0.0113	0.0319	
3-A	0.0117	0.0113	0.0236	101
3-B	0.0122	0.0113	0.0234	
4-A	0.0104	0.0037	0.0138	101
4-B	0.0101	0.0037	0.0147	
5-A	0.0145	0.0037	0.0184	103
5-B	0.0144	0.0037	0.0193	
6-A	0.0088	0.0037	0.0132	105
6-B	0.0087	0.0037	0.0132	
7-A	0.0295	0.0037	0.0342	99
7-B	0.0299	0.0037	0.0342	
8-A	0.0094	0.0040	0.0125	94
8-B	0.0091	0.0040	0.0123	
9-A	0.0221	0.0040	0.0266	101
9-B	0.0228	0.0040	0.0270	
10-A	0.0032	0.0040	0.0072	98
10-B	0.0034	0.0040	0.0071	
11-A	0.0074	0.0040	0.0104	92
11-B	0.0072	0.0040	0.0102	
12-A	0.0064	0.0040	0.0108	104
12-B	0.0064	0.0040	0.0110	
13-A	0.0057	0.0040	0.0100	97
13-B	0.0056	0.0040	0.0100	
14-A	0.0073	0.0040	0.0112	100
14-B	0.0072	0.0040	0.0116	
15-A	0.0064	0.0040	0.0108	102
15-B	0.0066	0.0040	0.0107	

correction (depending on the depth at which the unknown was read). Expressed as indican, this correction is a constant and does not restrict the depth at which readings may be made. The calculations give 0.0009 mg. as the indican equivalent of the

pigment solvent; this amount must be subtracted from the value arrived at as the absolute amount of indican present in the urine analyzed.

In Table II findings are given that establish the reliability of cobalt sulfate solution as a standard for the quantitative determination of indican. Under test conditions 1.5 per cent cobalt

TABLE IV

*Indican Determination in 24 Hour Urine Samples from Apparently Normal Individuals*

Sample No.	Urine volume	Sp. gr.	Indican found
	cc.		mg.
1	925	1040	148.0
2	825	1008	39.6
3	1180	1014	89.7
4	1600	1014	160.0
5	630	1040	85.7
6	1272	1017	106.5
7	635	1025	77.2
L. D., Mar. 9	1132	1017	77.0
" " 11	900	1020	82.8
" " 15	1040	1017	66.5
" " 16	1180	1017	70.8
" " 17	1350	1016	64.5
" " 25	1320	1017	58.1
" " 26	1150	1015	36.8
" " 29	1210	1018	77.4

sulfate in water is equivalent to 0.0086 mg. of indican. The method of calculation for urine indican follows:

$$\text{Mg. indican per 0.1 cc. urine} = \frac{0.0086 \times \text{reading of standard (mm.)}}{\text{reading of unknown (mm.)}} - 0.0009$$

If dilution to more than 2 cc. is necessary to bring the final color within optimum reading range, further dilution is made with glacial acetic acid. Since acetic acid offers no interference to light transmission, the correction needed is reduced in proportion to this dilution; thus, if the final dilution is 4 cc., only 0.00045 mg. need be subtracted from the value determined; if the dilution is 5 cc., only 0.00039 mg. need be deducted, etc.

Table III gives the results of indoxyl determinations on different

samples of fresh urine with and without the addition to each of definite amounts of standard indican solution.<sup>3</sup> The results show that satisfactory recovery can be made. It was intended that mixtures of urine and indican solution should be made so that in some of them most of the indican was derived from urine and *vice versa*. It may be noted that whatever the source of the major portion of the indican, the recovered total represents about the same percentage of the calculated total.

It appears from Table IV that this method reveals indican excretion in urine for 24 hour periods many times in excess of what has been reported as found by other methods, including that of Jolles. The reputed 24 hour average maximum output (40 mg.) is here a measure of minimum excretion. Still, the available ethereal sulfates are more than ample to bind these larger quantities of indoxyl (4).

#### *Comment*

Fearon and Thompson (5) have recently pointed out that pigments of red-blue shades may be obtained from the condensation of indoxyl with various phenols unsubstituted in the para position. We found that phenol or resorcinol could be substituted for thymol as a reagent in this test, but the latter offers the advantage of providing more intense pigments.

The introduction into the combination of reagents of conditions for the production of a fixed volume of an organic solvent for the pigment produced, which serves, as well, to extract the pigment, increases the reliability of the method as a quantitative one in obviating the inaccuracies attending the customary procedure of quantitatively removing the pigment by shaking with a solvent.

Potassium persulfate is introduced as a reagent since it prevents pigment formation with skatole under the conditions of the method. Omission of this reagent produces a pigment of redder hue, which on determination differs negligibly, if at all, from the

<sup>3</sup> Our indican crystals (furnished by E. Merck, Darmstadt, Germany) were purple, indicating a surface oxidation to indigo. However, they were readily soluble in water, giving a colorless solution. Toluene removed from the solution a faint blue pigment, but analyses of these pure solutions before and after shaking with toluene give identical values for indican.

bluer pigment resulting from its use as a reagent.<sup>4</sup> Very evidently the character of the oxidizing mixture used in the pigment production influences the hue of red-blue produced, though not the intensity of red contained therein; for it is primarily the intensity of red that is measured through the color filter used.

The absolute specificity of a color reaction in so complex a mixture as urine is difficult to establish, but we have so prepared the conditions of this test as to render most probable its specificity as a measure of indoxyl. This is chiefly accomplished by establishing those conditions under which indoxyl produces the most intense condensation pigment per unit of indoxyl and applying those conditions to minimal amounts of urine. Optimum reaction conditions allow the identification and determination of less than 0.0025 mg. of indican and permit the use of as little as 0.1 cc. of urine as an optimum quantity for indoxyl determination.

The method described in this paper has been adapted to the quantitative determination of indoxyl compounds in blood and will be presented soon.

#### SUMMARY

A method for the quantitative estimation of urinary indoxyl compounds is presented. Estimation of 24 hour indican excretion gives results several times higher than heretofore reported, 40 to 150 mg.

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<sup>4</sup> Apparently skatole exists in immeasurably small amounts in 0.1 cc. of urine.



# ON THE INFLUENCE OF VITAMIN B AND OF IODINE ON THE CALCIUM AND PHOSPHORUS METABOLISM OF RABBITS WITH HYPERPLASTIC THYROIDS

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Rabbits, when kept on a diet consisting principally of cabbage, develop simple goiter (1, 2). The possibility that a lack of vitamin B might facilitate the formation of thyroid hyperplasia was suggested by the fact that steaming the cabbage increased its goitrogenic activity (2). The heat-labile vitamin B might have been destroyed to a large extent by the process. During a study of the metabolism of rabbits in which thyroid hyperplasia had been produced by cabbage feeding (3), a definite influence was observed on the calcium and phosphorus metabolism when vitamin B was added to the diet. The reaction was the same as when iodine was given, namely an increase in the calcium excretion, resulting in a striking change in the ratio of calcium and phosphorus retention.

The supposition that vitamin B is concerned with thyroid metabolism has lately begun to gain recognition. Plimmer (4) in his studies on the vitamin B requirement of fowl and rats states that, "vitamin B appears to have a rôle not only in general metabolism, but also a special rôle in relation to the endocrine organs." The assumption of a relationship between vitamin B and thyroid metabolism is supported by the work of Cowgill and his coworkers (5), who present evidence that the amount of vitamin B required by the organism is determined chiefly by its caloric requirement. The vitamin B requirement of a given animal is significantly greater when the caloric factor is increased, as in hyperthyroidism (6).

Since we had noted on analysis of the cabbage used in the experiments mentioned above that the ratio of calcium and phosphorus in the food intake was  $\text{Ca:P} = 1:0.8$  in the experi-

mental period, when goiter was produced by feeding steamed cabbage, the question was raised whether iodine and vitamin B would act similarly on rabbits kept on a standard diet of alfalfa hay and oats if the ratio of Ca:P in the food were the same as in the diet of steamed cabbage.

#### EXPERIMENTAL

Adult male rabbits were used in all experiments. Five rabbits were kept for a period of 9 weeks on a vitamin B-deficient diet of autoclaved alfalfa hay and autoclaved rolled oats, with additions of calcium lactate and dibasic potassium phosphate, in order to bring the calcium and phosphorus intake to the desired level of  $\text{Ca:P} = 1:0.8$ . The ratio of calcium and phosphorus retention during this period was  $\text{Ca:P} = 1:1$  or less. The rabbits were then operated upon and the thyroids were observed to have increased in size and to present a very vascular appearance. Two rabbits now received 5.2 mg. of potassium iodide intraperitoneally, and three rabbits daily doses of vitamin B, two in the form of 2 gm. of Vitavose (Squibb) and one in the form of 2 gm. of dried yeast (Standard Brands Incorporated). Both vitamin B preparations were reported to be free from iodine. When Vitavose or yeast was given, the Ca and P intake was kept constant by deducting the amount of Ca and P contained in these materials from the supplementary Ca and P supplied by Ca lactate and dibasic potassium phosphate. After 4 weeks another operation was performed which showed the thyroids of the iodized rabbits to have undergone involution, and the glands of the animals which had received vitamin B to have somewhat decreased in size and to be flaccid in appearance, but still hyperplastic. In the rabbits which had received iodine the Ca excretion increased to such a degree during the 3 weeks following the injection that the ratio of Ca and P retention changed from  $\text{Ca:P} = 1:0.3$  to  $\text{Ca:P} = 1:216$ . During the next 2 weeks the Ca excretion decreased, though the Ca balance still remained negative, then increased again. The Ca excretion and the ratio of Ca and P retention thus proceeded in wave-like cycles, increases followed by decreases (see Table I).

When vitamin B was given, this form of the curve was even more striking. The Ca excretion rose to a high point during the 3

TABLE I  
Influence of Iodine on Calcium and Phosphorus Metabolism of Rabbits with Hyperplastic Thyroids. Daily Averages

Date	Weight gm.	Urine cc.	Feces gm.	Nitrogen								Calcium				Phosphorus				Ca:P	
				Total N				Intake gm.	Retention gm.	Intake mg.	Urine mg.	Feces mg.	Total excretion mg.	Retention mg.	Intake mg.	Urine mg.	Feces mg.	Total excretion mg.	Retention mg.	Intake mg.	Retention
				Intake gm.	Urine gm.	Feces gm.	Excretion gm.														
1932																					
Apr. 18-24	1630	80	20	1.3	0.6	0.5	1.1	0.2	403	43	312	355	48	321	79	226	305	16	1:0.81:0.3		
" 25-May 1	1640	90	20	1.3	0.5	0.5	1.0	0.3	404	42	312	354	50	321	80	225	305	16	1:0.81:0.3		
May 2-8	1660	90	20	1.3	0.5	0.5	1.0	0.3	401	53	318	371	30	320	81	229	310	10	1:0.81:0.3		
" 9-15	1680	80	20	1.3	0.6	0.4	1.0	0.3	400	41	313	354	46	320	84	221	305	15	1:0.81:0.3		
" 16-22, operated on	1690																				
" 23-29*	1720	60	10	1.0	0.7	0.2	0.9	0.1	352	66	206	272	80	231	72	118	190	41	1:0.71:0.5		
" 30-June 5	1570	100	10	1.4	0.9	0.3	1.2	0.2	415	115	328	443	-28	334	83	189	272	62	1:0.81:50		
June 6-12	1600	90	10	1.4	0.7	0.4	1.1	0.3	415	124	469	593	-178	334	53	243	296	38	1:0.81:216		
" 13-19	1680	110	10	1.4	0.9	0.3	1.2	0.2	415	110	316	426	-11	334	68	214	282	52	1:0.81:63		
" 20-26, operated on	1780	100	10	1.4	0.7	0.4	1.1	0.3	415	107	317	424	-9	334	56	234	290	44	1:0.81:53		
" 27-July 3	1820	110	10	1.4	0.7	0.4	1.1	0.3	415	78	416	494	-79	334	61	253	314	20	1:0.81:99		
July 4-10	1830	110	10	1.4	0.8	0.4	1.2	0.2	415	68	427	495	-80	334	63	219	282	52	1:0.81:132		
" 11-17	1840	110	10	1.3	0.8	0.3	1.1	0.2	414	57	420	477	-63	328	76	243	319	9	1:0.81:72		

\* During the week of May 23 to 29, 5.2 mg. of potassium iodide were administered in two doses.

TABLE II  
*Influence of Vitamin B on Calcium and Phosphorus Metabolism of Rabbits with Hyperplastic Thyroids. Daily Average*

Date	Weight gm.	Urine cc.	Feces gm.	Nitrogen								Calcium				Phosphorus				Ca:P																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
				Total N				Intake gm.	Urine gm.	Feces gm.	Excretion gm.	Retention gm.	Intake mg.	Urine mg.	Feces mg.	Total excretion mg.	Retention mg.	Intake mg.	Urine mg.	Feces mg.	Total excretion mg.	Retention mg.	Intake	Retention																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
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\* Beginning with May 23, 2 gm. of Vitavose were administered daily.

weeks after the administration, changing the ratio of calcium and phosphorus retention from  $\text{Ca:P} = 1:1$  to  $\text{Ca:P} = 1:226$ , and from  $\text{Ca:P} = 1:0.3$  to  $\text{Ca:P} = 1:177$ , respectively. During the following 2 weeks the calcium excretion fell, bringing the ratio of calcium and phosphorus retention to within the normal range, then rose again, and ultimately fell to the normal level (see Table II).

In order to decide the question whether vitamin B really was the active agent which is responsible for the changes in the calcium and phosphorus metabolism, control experiments were run on another group of rabbits. Autoclaved yeast and autoclaved Vitavose were substituted for yeast and Vitavose as used in our previous experiments (presumably as sources of vitamin B). No change in the Ca excretion occurred and the ratio of retention of calcium and phosphorus remained unchanged,  $\text{Ca:P} = 1:1$ .

Total nitrogen (Kjeldahl), calcium (volumetric), and phosphorus (Fiske-Subbarow) were determined in the food, Vitavose, and yeast, in the dried feces in weekly lots, and in the urine in 3 and 4 day lots. Tables I and II show daily averages for weekly periods. The ratio of  $\text{Ca:P} = 1:0.8$  in the food intake was kept constant throughout the experiment. pH was determined weekly and remained constant at 8.2 to 8.4, which is normal on a diet of hay and oats.

#### DISCUSSION

It has been shown as early as 1912 that thyroid feeding increases the calcium excretion in rabbits (7), and from the work of Aub and coworkers (8) and others (9) it is well known that increased thyroid secretion causes a striking accentuation of the calcium excretion. Aub suggests that a direct stimulating catabolic effect on the calcium deposits in the bones seems the most likely explanation.

In the experiments reported in this communication we found that in rabbits kept on a diet poor in vitamin B, with a ratio of calcium and phosphorus in the food intake of  $\text{Ca:P} = 1:0.8$ , addition of iodine and vitamin B produced the same striking change in the calcium and phosphorus metabolism as in the cabbage-fed animals. The excretion of calcium rose to an extremely high level and the ratio of retention of calcium and phos-

phorus changed from  $\text{Ca:P} = 1:1$  or less to  $\text{Ca:P} = 1:226$  at the peak of the reaction. The thyroids became increasingly hyperplastic on the vitamin B-deficient diet, which is in accordance with the fact that goiter has been produced in rats on a vitamin B-deficient diet (10). But the well known effect of iodine—*viz.* causing involution of the hyperplastic thyroid—was paralleled only in direction but not in degree by the addition of vitamin B to the diet, which resulted in a gland of flaccid appearance, but which had only slightly decreased in size.

The fact that vitamin B and iodine were found to influence the calcium and phosphorus metabolism in the same direction under various experimental conditions would seem to suggest that both iodine and vitamin B act through the same mechanism. It has been adequately demonstrated that the utilization of iodine in the body is dependent on the thyroid gland. Vitamin B may produce its effect on calcium and phosphorus metabolism either indirectly through the thyroid gland in a manner similar to that of iodine or it may influence an intermediary mechanism. While a lack of vitamin B facilitates thyroid hyperplasia, additions of vitamin B to the diet of rabbits with hyperplastic thyroids cause only slight changes in the anatomical condition of the gland. It seems probable that the action of vitamin B, though exactly the same as that of iodine on the calcium and phosphorus metabolism, and while possibly involving the thyroid, is dependent, as well, on some other mechanism.

#### SUMMARY

Vitamin B and iodine influence the calcium and phosphorus metabolism of rabbits with hyperplastic thyroids in the same direction. A vitamin B-deficient diet facilitates the development of thyroid hyperplasia in rabbits.

Administration of either vitamin B or of iodine causes an increase in the calcium excretion and a striking change in the ratio of calcium and phosphorus retention.

The assumption that the active agent present in yeast and Vitavose which influences the calcium and phosphorus metabolism of rabbits with hyperplastic thyroids is vitamin B is supported by the finding that autoclaved yeast and autoclaved Vitavose show no influence on the calcium and phosphorus metabolism.

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## FATTY ACIDS OF LIVER LECITHIN

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The phospholipids, along with cholesterol, are now recognized as essential elements of tissues, while the characteristic constituents of the phospholipids are the fatty acids. In any study of the metabolic activity of tissues the nature and proportions of the various fatty acids in the phospholipids therefore become important and have been the subject of increasing interest and speculation. Because of its many sided activity in metabolism, the liver has received a large share of attention and more work has been done on its lipid constituents than on those of any other organ.

In the following work an attempt is made to find out the nature of the fatty acids and the percentage distribution in liver lecithin with special reference to the unsaturated acids. Since these results were first reported (1) an outstanding piece of work by Klenk and Schoenebeck (2) has appeared dealing with the fatty acid distribution in the total ether-soluble phospholipid (lecithin plus cephalin) of liver, obtained by the method of fractional distillation, to which reference will be made.

The occurrence in liver of fatty acids not found in ordinary fat was first noted by Hartley (3) who described the 4 double bond  $C_{20}$  acid now known as arachidonic. Since that time the presence of this acid in various tissues has been established by many investigators. Levene and Rolf (4) in 1922 found it in egg yolk. Bloor (5, 6) showed its presence in varying amounts in liver, kidney, pancreas, lung, and in heart, jaw, diaphragm, neck, and round muscles. Brown (7) identified arachidonic acid in the thyroid, suprarenal glands, and spleen. In liver he believed that it was the only highly unsaturated acid, a finding which has since been disputed (2). It was found by Eckstein (8) and Ellis and Zeller (9) in

small amounts in the body fat. Wesson (10), on the basis of its occurrence and variations in tissues under changing conditions, gave it a leading place in the metabolism of fat. Hartley (3) found another acid in pig liver which has not been found elsewhere and which he thought was important in the consideration of the desaturating power of the liver, an acid isomeric with ordinary oleic acid.

The distribution of the fatty acids in the lipids of liver or any organ has not been closely examined. Hartley (3) showed the presence of palmitic, stearic, oleic, linoleic, and arachidonic acids in liver. Brown (11) found these acids plus more highly unsaturated ones in brain, as did Rudy and Page (12). Higher acids than  $C_{20}$  were not found in liver, however, until recently, when Klenk and Schoenebeck (13, 2) reported a  $C_{22}$  acid with five double bonds. Bloor (5, 6), Brown (14), and Theis (15) found no evidence of a  $C_{22}$  acid in the bromine addition products. Brain and liver apparently contain much larger amounts of the more highly unsaturated and long chain acids than any other organ. The fact that the liver has a large proportion of unsaturated acid could be accounted for in part by the theory of Leathes and Meyer-Wedell (16), according to which the fatty acids are desaturated by this organ. This theory, however, does not answer the question as to how the long chain acids originate. Hartley (3) stated that there is not enough arachidic acid in the body to account for the large amount of arachidonic acid found. Klenk and Schoenebeck (2) raise this point in their discussion of Leathes' hypothesis of fatty acid desaturation by the liver.

Hartley (3) had another idea, based on the data of Magnus-Levy (17), Leathes (18), and himself (3), that the highly unsaturated acids are formed directly from carbohydrates. He found that animals kept on a "fat-free" carbohydrate diet produced liver fatty acids with a high iodine number. Wesson (10), experimenting with normal, fasting, and phlorhizinized rats, found that his data did not support Hartley's idea (3). In fasting animals, where there would be an active fat but a subnormal sugar metabolism, the amounts of arachidonic acid were increased. He concluded that arachidonic acid was an intermediary product formed in the metabolism of fatty acids having less than 20 carbon atoms. Powell (19) fed

tricaprylin and trilaurin to rats and found in the body fats longer chain acids than those fed. This work indicated an ability of the organism to form longer chain acids from shorter ones.

#### EXPERIMENTAL

*Preparation of Lecithin*—Fresh liver tissue is freed from visible fat, finely ground in a meat chopper, and treated as described by Bloor (20), briefly as follows: 1000 gm. of finely ground tissue are dehydrated in 3 liters of 95 per cent alcohol for 1 to 2 hours at 36°, the alcohol is filtered off, and the tissue extracted for 3 hours with fresh alcohol. The combined alcohol is distilled under reduced pressure and the residue taken up in petroleum ether (Bloor used ordinary ether). The petroleum ether is concentrated to a syrup and poured into 100 cc. centrifuge tubes in 25 cc. portions. The phospholipid is precipitated by addition of 75 cc. of acetone to each tube, the precipitate is thoroughly kneaded with acetone, redissolved in petroleum ether, and the reprecipitation and kneading repeated three or four times. Sphingomyelin,<sup>1</sup> being insoluble in petroleum ether, appears as a precipitate and is centrifuged out at the various times when the phospholipid is dissolved in petroleum ether. Cephalin is separated from the lecithin by precipitation with absolute alcohol. After the cephalin is removed the alcohol containing the lecithin is distilled under reduced pressure, the lecithin dissolved in petroleum ether, and aliquots taken for weight and iodine numbers.

*Saponification and Extraction of Fatty Acids*—The lecithin is saponified by boiling 5 or 6 hours with 16 to 20 gm. of stick sodium hydroxide dissolved in 200 cc. of 50 per cent alcohol. The fatty acids are extracted with petroleum ether after acidification with hydrochloric acid. Weight and iodine numbers are determined in aliquots. To the petroleum ether<sup>1</sup> solution (200 cc.) are then added 3 or 4 volumes of acetone, which cause the formation of a flocculent precipitate which is centrifuged out (stoppers being needed, as the precipitate is so light that otherwise it will not settle). The petroleum ether and acetone are distilled under a vacuum and the fatty acids dissolved in 95 per cent alcohol.

*Lead Soap Separation of Liquid and Solid Fatty Acids (Modified*

<sup>1</sup> See "Notes on procedure."

*Twitchell Method*)—To the boiling alcoholic solution of **fatty acids** (100 cc.) are added 100 cc. of boiling alcohol containing **lead acetate** in excess over the amount calculated for the solid acids. The solution is allowed to cool slowly and then to stand at a temperature maintained constant at 15–20° overnight. The next morning the mixture is stirred and the solid lead soaps are centrifuged down. The solid lead soaps are thoroughly extracted with **petroleum ether** to remove the “solid unsaturated acid.” The washings are evaporated and the residue is refluxed for  $\frac{1}{2}$  to  $\frac{3}{4}$  hour with **about 20 cc.** of 95 per cent alcohol containing 1 drop of glacial **acetic** for each 20 cc. of alcohol. The boiling alcohol, which contains any remnants of liquid unsaturated and solid saturated acids, is poured off, cooled slowly, and set away for 3 or more hours at **about 17°**, for the solid soaps to precipitate out, after which the **standard** procedure as described below is followed.

The residue (containing the so called solid unsaturated acid) which remains insoluble after refluxing with alcohol, is dissolved in ether, treated with 10 per cent hydrochloric acid (the precipitated lead chloride being thoroughly extracted with ether), and the ether extracts washed free from inorganic acid and lead. If emulsions form upon addition of water, these emulsions must be drawn off, warmed on a steam bath, and, upon melting, the fatty acid layer is extracted with ether; otherwise there is a considerable loss of fatty acids.<sup>1</sup> After drying and weighing, iodine number and melting point are determined.

The lead soaps of the solid acids are suspended in ether and decomposed with 10 per cent hydrochloric acid, washed free from acid and lead, dried, weighed, and the iodine number and melting point determined. The liquid acids are freed in the usual manner (*Twitchell* (21)), dried over sodium sulfate, made up to a volume of 200 cc., and aliquots taken for weight and iodine number.

*Bromination of Liquid Fatty Acids*—If carefully purified petroleum ether<sup>1</sup> has been used for the extraction of the liquid acids the bromination may proceed at once.

Approximately 5 to 6 gm. of fatty acids are dissolved in 200 cc. of petroleum ether. After cooling to a temperature of –5°, bromine is slowly added drop by drop from a burette (the flask being shaken constantly) until a dark orange or light red color is obtained. About 10 per cent excess of bromine is required. After standing

in the refrigerator for at least 15 hours the precipitate is centrifuged down and the excess bromine and the dibromide are washed out with petroleum ether.

*Separation of Dibromide*—The petroleum ether-soluble fraction, which contains the dibromide and excess bromine, is immediately placed in a pressure flask (500 cc.) and the petroleum ether and excess bromine are distilled under reduced pressure with only heat enough to prevent the formation of ice on the flask. Additional petroleum ether is added once or twice to the residue, which is a light yellow oil, and the procedure repeated to insure complete removal of the excess bromine. The oil is redissolved in petroleum ether, transferred to a small weighed flask or beaker, and the petroleum ether concentrated to a volume of 15 cc. by distillation in a vacuum desiccator, in which a capillary tube, attached to a stop-cock, reaches to the bottom of the liquid and serves to prevent foaming or bumping. The solution of dibromide is then allowed to stand in the refrigerator for several days.<sup>1</sup> A white precipitate separates out in the petroleum ether which has been found to be tetrabromide. The precipitate is centrifuged out and the dibromide dried in the desiccator, weighed, and the bromine content determined according to the method of Brown and Beal (22).

*Separation of Tetrabromide*—The petroleum ether-insoluble fraction, which contains the tetra- and octabromides, is treated with chilled, peroxide-free, dry ether to extract the tetrabromide which is ether-soluble. About 150 to 200 cc. of ether are used in 50 cc. portions. The ether is centrifuged until clear and placed in a weighed flask or beaker and the ether removed by vacuum distillation in the desiccator as previously described. It is allowed to remain in the desiccator for some time with the pump running to remove any excess bromine. It is then dissolved in 15 cc. of dry ether and set away in the refrigerator for a few days. In this fraction some octabromide is dissolved, which, upon chilling, precipitates out. The rest of the procedure is the same as for the dibromide.

*Separation of Octabromide*—The ether-insoluble fraction, containing the hexa- and octabromides, is treated with 50 cc. portions of boiling benzene. Better extraction is obtained if the bromides are powdered, vigorously boiled in the benzene, and centrifuged

as quickly as possible, as the solubility in even boiling benzene is very small.

The benzene-insoluble fraction contains the octabromide (together with some bromides of more highly unsaturated acids). This fraction is dried and weighed and bromine content determined.

*Iodine Number*—Three methods were used to measure the degree of unsaturation of the fractions: the Hanus method, the Rosenmund-Kuhnhehn pyridine-dibromide method (23), and the rhodanate or thiocyanogen method of Kaufmann (24).

#### *Notes on Procedure*

*Sphingomyelin* It is well known that sphingomyelin is removed with difficulty from lecithin and cephalin although it is insoluble in ether and petroleum ether. A certain amount is held in solution by the soluble phospholipids. Although, as noted above, attempts are made throughout the separation of the phospholipid from neutral fat to remove the sphingomyelin, there is evidence that all is not taken out.

Following the procedure described by Sinclair (25) a precipitate was found to form upon addition of an excess of acetone to the mixed fatty acids. Like that found by Sinclair, it gave a red color with sulfuric acid, which is a property of cerebrosides. Further properties similar to those of sphingomyelin and cerebrosides were displayed by this substance. It is somewhat soluble in cold pyridine, more soluble in warm pyridine, partly soluble in ether and petroleum ether, and difficult to saponify with alkali. Not only has it been found in the mixed fatty acids but also in the liquid acid fraction and in the emulsions formed in the solid unsaturated acid fraction (see "Discussion").

*Lead Soap Separation of Liquid and Solid Acids*—Various workers have found in using the Twitchell lead soap method (21) for the separation of liquid and solid acids in animal tissue, that it is difficult to recover 100 per cent of the fatty acids. It has been observed that upon addition of boiling alcoholic lead acetate to a boiling alcoholic solution of fatty acids a sticky mass, commonly referred to as pitchy residue, is formed. After the lead soaps have stood in the cold overnight and the alcohol and solid lead soaps have been removed, the sticky residue has been found to remain in the bottom of the flask. It will not dissolve in boiling

alcohol, but is soluble in ether and can be decomposed with nitric or hydrochloric acid. The freed fatty acids are washed only with difficulty, because tenacious emulsions are formed upon addition of water. These emulsions are also encountered in both the liquid and solid acid fractions and their formation is apparently related to the presence of the pitchy residue.

Attempts were made to prevent the formation of this pitchy residue by varying the temperatures of the alcoholic solutions upon the addition of one to the other. At a temperature ranging between 50–55° it was found that no pitchy residue formed, but traces formed if the solid lead soaps were treated with boiling alcohol for their reprecipitation. When, however, there was no pitchy residue observed, the emulsion formation was increased tremendously in the liquid and solid fatty acid fractions. These emulsions, when drawn off and warmed on a steam bath, cleared fairly well. Upon extracting with ether it was found that between 10 and 15 per cent of the solid acids was recovered from them. An absolute recovery, however, seems almost impossible, for the acids have to be washed free of inorganic acid and the emulsions reform, carrying with them some fatty acid.

The method of Cocks, Christian, and Harding (26) for the extraction of solid, unsaturated acids was tried with the hope that it might help with the difficulties in the recovery of solid and liquid acids. The method, with modifications, has been described in detail above. It was found that if sufficient lead had been added to precipitate all the solid acids and if the solid lead soaps were thoroughly washed with petroleum ether that there would be no emulsions formed except in the solid, unsaturated fatty acid fraction. If the emulsions are carefully handled 93 to 95 per cent recovery can be made, apparently the greatest loss being incurred by failure thoroughly to extract the emulsions; at the best, it is a tedious task.

*Bromination of Unsaturated Acids*—The methods for the separation of the unsaturated acids by their bromination products are unsatisfactory. One difficulty is the lack of specific solvents for any one of the bromides. For instance, the solvent used for the separation of the hexabromide is not a good one. The hexabromide is only slightly soluble in warm benzene, while on the other hand some of the octabromide, which is slightly soluble in benzene, is carried along.

When fatty acids were brominated in ether difficulty was encountered in freeing the ether-soluble bromides from the excess bromine. The method of washing it out with sodium thiosulfate, as described by Ellis and Isbell (27) did not prove satisfactory, as a brown tarry mass resulted. In the present work removal of the solvent and excess bromine was accomplished by distillation under reduced pressure as described above, the bromination being carried out in petroleum ether instead of ether. Special care was found necessary in the preparation of the petroleum ether for use. Many samples contain unsaturated hydrocarbons, which, when brominated, interfere with the separation of the fatty acid bromide. The difficulty was finally solved by the finding of a sample of petroleum ether (Skelly Oil Company) which could be easily purified. The material was fractionally distilled and only that fraction used which boiled below 60°. This product had an iodine number of 4.6. It was allowed to stand over sulfuric acid, with occasional shaking, then distilled over barium hydroxide, and the iodine number was reduced to 1 or less.

The di- and tetrabromides, as first prepared, gave values for bromine of 10 to 14 per cent too high. This dibromide was redissolved in petroleum ether and allowed to stand in the refrigerator for a few days, when a precipitate separated out which proved to be a tetrabromide compound. The values of the soluble portion were still somewhat high for the dibromide but it is possible that there are isomers of the more highly unsaturated acids which are both ether- and petroleum ether-soluble and similar to those described by Rudy and Page (12). The tetrabromide fraction, after standing in ether in the cold for several days, gave a white precipitate. With this precipitate (which on analysis was found to be octabromide) removed, the percentages were greatly reduced. This procedure for both di- and tetrabromides is now routine.

The bromides, as finally obtained, are of a light yellow color, the dibromide being an oil and the tetrabromide generally a flaky mass which can be powdered. In some cases, however, the tetrabromide is a sticky, dark colored substance, which is difficult to handle in the determination of the bromine content.

*Comparison of Hanus and Rosenmund-Kuhnhehn Methods for Iodine Number*—The Hanus solution, containing bromine, iodine, and acetic acid, cannot be used for the determination of iodine

values of cholesterol, as it causes substitution as well as addition. Yasuda (28) examined the Rosenmund-Kuhnnehn solution (23), which consists of pyridine, bromine, and acetic and sulfuric acids, and found that it gave theoretical values for cholesterol and slightly lower values for ricinoleic and oleic acids than did the Hanus solution.

In our work values for each solution were determined for the various fractions of liver lipids and in every case the pyridine values were lower than the Hanus. The averages for intact lecithin, for example, were P 88, H 91, for mixed fatty acids P 121, H 126, for liquid fatty acids P 209, H 214 (Table I, Series I). Values obtained for oleic acid, Kahlbaum's K brand, were 90 for the pyridine method, while the Hanus method gave 93. Whether this higher value for the Hanus method is due to a slight tendency toward substitution cannot be definitely stated but the more unsaturated the acids the greater the difference in the two methods. Yasuda (28) found a difference of 2.8 for oleic acid, 1.7 for ricinoleic acid, and 6 for cod liver oil. In our work on lecithin there was an average difference of 3 for intact lecithin, 5 for mixed fatty acids, and 5 for unsaturated acids.

*Thiocyanogen Number, Rhodanate Number*—Kaufmann (24) found that thiocyanate dissolved in a solution of bromine in water-free acetic acid reacts with unsaturated fatty acids to a certain extent like bromine and iodine. Using various fatty acids and oils he obtained the following results, which he compared with the the iodine values obtained on the same substances by converting the thiocyanogen or rhodanate number to the iodine equivalent.

Fat	Iodine No.	Rhodanate iodine No.
Oleic acid .....	89.9	89.9
Linoleic " .....	181.09	90.545
Palmitic " .....	0	0
Olive oil .....	80.8	76.6
Peanut " .....	87.1	78.5

It is apparent from these results that only the bond in position  $\Delta^9-10$  adds thiocyanate. In later work, however, he found that it added to two bonds in linolenic acid (29).

The time needed for this reaction to take place is much greater

than for the addition of iodine or bromine as by the Hanus procedure. Kaufmann found that 5 hours was a sufficient length of time for a value of 90 to be obtained for oleic acid. However, at the end of 5 hours the highest value obtained in this laboratory was 84, for an oleic acid which with the pyridine and Hanus solutions gave values of 90 and 93 respectively. After allowing a sample to stand 84 hours Dr. H. C. Hodge of this laboratory obtained a value of 90. After 5 hours linoleic acid gave a value of 108, cod liver oil 99, and linseed oil 102. Zeleny and Bailey (30), working with the fatty acids of lard, found that it was necessary to let the sample stand for 17 hours before titrating.

#### DISCUSSION

*Fatty Acids*—Table I, Series I, shows the averages of several analyses of three different lots of lecithin (kindly sent to us by Dr. H. Gregg Smith, State University of Iowa), used primarily for the perfection of the procedure. In Series II of Table I are given the data obtained from seven fresh beef livers. On the whole the values are not very different in the two series. In Series II the weight of lecithin is per 1000 gm. of moist liver. The recovery of mixed fatty acids is 66 to 67 per cent respectively of the intact lecithin, which is 93 per cent of the theoretical value for the fatty acids in an oleyl-stearyl lecithin and about three points higher for palmityl-oleyl lecithin. The iodine values in both series are considerably higher than those found previously by Bloor (5). The average for the intact lecithin in his work was 83 as compared with 90 and 91 in the present—the Hanus figures being used in both cases. The difference may probably be referred to diet since Sinclair (25, 31) has shown that diet influences the unsaturation of the phospholipids. Correspondingly higher values are also found for the liquid acids, 213 as compared with 160 in the earlier work, while the percentage of liquid acids in this work is somewhat higher—averaging 49 as compared with 40. These differences are due in part, at least, to the improvement in technique in the separation of liquid and solid acids.

Calculated from the figures of liquid acids in per cent of mixed acids the iodine numbers of the liquid acids are about 30 per cent too low. Two possible causes present themselves: (1) The low percentage of recovery of the solid, liquid, and solid unsaturated

TABLE I  
*Lecithin of Beef Liver.*

	Lecithin			Mixed fatty acids			Liquid fatty acids			Solid fatty acids			Solid unsaturated acid	Recovery		
	Weight gm. moist liver per 1000	Iodine Nos.			Lecithin	Iodine Nos.			Mixed fatty acids	Iodine Nos.		Mixed fatty acids			Melting point	
		Hanus	Pyridine dibromide	Rhodanate iodine		Hanus	Pyridine dibromide	Rhodanate iodine								
Series I																
Sample 1 (2).....		88	87	65	65	124	125	72	53	212	208	108	35	661-62	94	97
“ 2* (5).....		93	87	68	66	126	120	72	48	213	207	107	34	661-62	94	87
“ 3 (6).....		91	89	64	67	128	118	70	48	217	211	112	37	562-63		90
Average.....		91	88	66	66	126	121	71	49	214	209	109	36	561-62	94	91
Series II																
Sample 1.....	24.1	93	87	58	67	127	124	66	51	203	178†		31	1261		90
“ 2.....	21.6	88	85		68		84	63†	179†	172†	108		33*	262	2†	98
“ 3.....	22.2	88	82	76	63	112	106	77	58	233	224	119	41	261	0†	99
“ 4.....	18.2	94	92	69	67	126	125	82	52	210	202	113	33	463	5	90
“ 5.....	22.5	90	82	66	66	130	119	68	48	231	217	108	39	261	10	97
“ 6.....	23.3	96	93		70	130	123		50	201	192§		32	6	76	88
“ 7.....	24.1	85	84		69	111	109		40	197	197		36	6	90	82
Average.....	22.3	90	86	67	67	121	119	77	49.8	212	206	111	35	562	92	92

The figures in parentheses show the number of analyses made of each sample.

\* In Sample 2, solid acid was found in the liquid acid fraction.

† Not included in average. ‡ "Substance X" removed. § Oxidation occurred.

acids—which might indicate the loss of a highly unsaturated acid during the separation. On the basis of the discrepancy between the theoretical and actual iodine number (Hanus) the percentage of loss would be 12, which is near the 10 per cent deficiency found. However, when the recovery is higher than 90 per cent there is still a discrepancy in the iodine number. (2) Oxidation during separation is the other and probably more likely cause for the low value. The iodine number of the solid unsaturated acid (which averages 5 per cent of the mixed fatty acids) is much lower than that of the liquid acids. As this fraction is removed, the liquid acids should have an iodine number higher than calculated, but actually it is lower, which again points to oxidation. Isomeric change in the fatty acid molecules with change in the readiness with which they absorb iodine is another possibility.

The averaged mean molecular weight of the liquid acid is 319, a figure which is close to the 312 obtained by Hartley. This high figure indicates three possibilities: (1) the presence of high molecular fatty acids, (2) oxidation, and (3) association compounds of some kind.

The solid acids as separated, average 36 per cent of the mixed fatty acids, have an iodine number of 5, a melting point of 63°, and a mean molecular weight of 276, which again agrees with that of Hartley, who obtained a molecular weight of 275 for the saturated acids. On the basis of the mean molecular weight, there would be a percentage proportion of stearic to palmitic acids of 71 to 29, which indicates that the saturated as well as the unsaturated fraction of phospholipid has longer chain acids than does the neutral fat.

*Analysis of Bromides. Fatty Acid Recovery*—The method most commonly used for the separation of the unsaturated acids has been that making use of the solubilities of their bromine addition products. In the present work, however, a great deal of difficulty was encountered in fractionating the bromides, especially in the ether and petroleum ether fractions, the bromine content of the material separated being far too high for the bromides of linoleic and oleic acids respectively (Table III). It was obvious that bromides of the more unsaturated acids must be present, probably as more soluble isomers, a conclusion which has been confirmed by the recent work of Rudy (32), who found that bromination does result in isomeric bromides of different solubility.

After considerable experimenting with calculations on different assumptions it seemed simplest and probably most correct (especially after the appearance of Rudy's work) to assume that the high percentage of bromine in the petroleum ether-soluble fraction is due to isomeric tetrabromides and the high value in the ether-soluble fraction to isomeric octabromides. The values in Table II, which show the distribution of the fatty acids, were obtained by calculation on this basis and under these conditions the total recovery of the various acids gave a value of 100 per cent or close to it in nearly every sample. The distribution of acids differed somewhat from that of Klenk and Schoenebeck (2) on the mixed phos-

TABLE II  
*Distribution of Unsaturated Fatty Acids*

Type of acid*	Per cent of liquid acids										
	Series I				Series II						
	Sample 1	Sample 2	Sample 3	Average	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Average
Arachidonic.....	32.0	28.4	30.0	30.0	20	27	30	32	40	35	31
Linoleic.....	44.3	45.0	45.0	44.7	46	55	46	47	28†	47	48
Oleic.....	25.0	19.6	27.0	23.9	34	8	20	10	16	21	18
Total recovery....	101.3	93.0	102.0	98.6	100	90	96	89	84†	103	97

\* These terms represent fractions, the exact composition of which is unknown. See discussion.

† Not included in average.

phatides of beef liver, obtained by a distillation procedure. Klenk and Schoenebeck found 28 per cent of the mixed fatty acids or 47 per cent of the liquid acids to be highly unsaturated  $C_{20}$  and  $C_{22}$  acids, and the rest to be  $C_{16}$  and  $C_{18}$  acids. Our data gave a different distribution; 30 per cent of the liquid acids was highly unsaturated, 45 per cent was linoleic acid, and 20 per cent oleic acid.

Theis (15), using the bromination method, obtained a value of 34 per cent for arachidonic acid, which is in close agreement with our figure.

*Ether-Insoluble Bromides*—It is in this fraction that our two series differ most (see Table III). In Series I the bromine content averages 67.6 per cent before treatment with benzene and 68.4 after. In Series II the values are lower—64.8 and 66.3, respec-

tively. The higher percentage in Series I suggests the possibility of a  $C_{22}$  5-bond acid which has a theoretical bromine content of 70.8 per cent. Klenk and Schoenebeck (13) found that the ether-insoluble bromide from beef liver gave the following analysis: C 24.7 per cent, H 3.5 per cent, and Br 68.2 per cent, which is too high for a  $C_{20}$  4-bond acid. The theoretical figure for a mixture containing 25 per cent of the bromine derivative of a  $C_{22}$  5-bond acid and 75 per cent of the bromine derivative of arachidonic acid would

TABLE III  
*Bromine Content of Bromides*

Fraction	Per cent of bromine						
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Series I							
A. Ether-insoluble bromide							
Before benzene.....	68.0	67.0	66.9				
After ".....	69.5	67.7	68.2				
B. Ether-soluble bromide	60.0	58.0	57.0				
C. Petroleum ether-soluble bromide.....	43.5	46.0	44.0				
Series II							
A. Ether-insoluble bromide							
Before benzene....	66.5	63.6	63.0	65.0	66.0		65.0
After ".....	68.0	66.3	64.0	68.0	67.5	66.2	64.5
B. Ether-soluble bromide		54	60.6	59.0	55.0	62.0	61.0
C. Petroleum ether-soluble bromide.....		45	47.0	48.0	45.0	45.0	46.5

be 68.5 per cent, which is very close to the above value found and indicates the presence of a  $C_{22}$  5-bond acid in this series of livers. In a more recent paper Klenk and Schoenebeck (2) isolated a  $C_{22}$  5-bond acid by means of distillation of the methyl esters.

*Benzene-Soluble Bromide*—Since the percentage of bromine of the benzene-insoluble fraction was higher than that of the total ether-insoluble bromide it indicates that the benzene removes a substance with a lower bromine content. The percentage of bromine in the benzene-soluble fraction is 65 to 66, which is too high for

hexabromide. It is probable then that this fraction is composed of octabromide and a small amount of tetrabromide. The possible presence of octabromide of higher molecular acids is not excluded.

**Solid Unsaturated Acid**—In the method of Cocks, Christian, and Harding (26) the solid lead soaps are extracted with petroleum ether to remove any solid or liquid unsaturated acids clinging to this fraction. The residue from the petroleum ether extraction is refluxed in boiling alcohol and the whole is normally soluble in hot alcohol. Following this procedure we found a fraction, insoluble in boiling alcohol, and it was separated from the rest and analyzed. It was similar in appearance to the pitchy residue found in earlier work (5) and was also ether-soluble. Upon acidification with 10 per cent hydrochloric acid, a brown, clear, semisolid substance was isolated. Its iodine number was 98, very close to that of elaidic acid and the substance was consequently referred to as a solid unsaturated acid.<sup>2</sup> A melting point determination was attempted but the material did not behave like pure fatty acid. It sintered at 45°, turned black between 50–60°, and at no time formed a true liquid.

After this fraction had been isolated from several samples of lecithin the separation of substance X by acetone from a petroleum ether solution of the mixed fatty acids, as described by Sinclair (25), was made and it was found that when a large amount of substance X precipitated out, the alcohol-insoluble fraction was very much decreased. The two substances as isolated were very different in appearance, the acetone precipitate (substance X) being a white or pale yellow flaky material, while the other was similar to a solid, unsaturated acid. Positive qualitative tests for nitrogen and phosphorous had been found for substance X and so quantitative analyses were made on the solid unsaturated acid. There was found a high percentage of nitrogen, averaging 7.6, while the phosphorous values averaged 2.8 per cent, making a P:N ratio of 1:6.

Some of substance X was then treated with hot alcohol and part was soluble and part remained insoluble. The soluble fraction contained 9.2 per cent nitrogen, while the insoluble part contained but a mere trace. Another sample was treated with lead acetate

<sup>2</sup> The term solid unsaturated acid is used as a convenience rather than with a specific meaning. The composition of the substance is unknown.

and the fraction corresponding to the solid unsaturated acid yielded an acid identical in appearance with it, but of a lower degree of unsaturation. Sinclair (personal communication) has found a similar material in his substance X.

In many respects the properties of the solid unsaturated acid resembled those of the pitchy residue acids. In appearance they were unlike, because the latter were contaminated with solid acids and for the same reason the iodine number was lower. The lead soaps of both were sticky, insoluble in cold and boiling alcohol, and soluble in ether. As acids, both formed thick emulsions with water; in fact their affinity for water was so great as to suggest hydroxyl groups and the material was accordingly acetylated (33). The pitchy residue acids gave values ranging from 140 to 149, while the solid unsaturated acid gave more divergent and lower values. Before the presence of nitrogen was found it was concluded that the acetyl value might be due to the presence of a hydroxyl group, but as the amino group also forms an acetyl derivative one cannot draw that conclusion, without further analysis.

Klenk and Schoenebeck (2) in their work on liver have reported a substance with a nitrogen content of 2.19 per cent and a phosphorous percentage of 3.34, giving the following ratio, P:N 1:1.49. This substance has a melting point of 156–157° and begins to sinter at 80°. This material was recrystallized from alcohol and pyridine and so undoubtedly was much purer than ours. They concluded that their product was lignoceryl sphingomyelin and split-products.

*Rhodanate Numbers*—Until more data have been accumulated concerning the action of rhodanate on the higher fatty acids, nothing can be said in regard to the significance of the rhodanate numbers obtained for liver lecithin. If this radical adds only to the one bond, then the values should not be higher than 90 for the liquid fatty acids; actually they average 111. These figures obtained for the mixed fatty acids (average 77) are not what would be expected by calculation from the liquid acids, nor are those of the intact lecithin (average 67) in accordance with calculations based on the mixed fatty acid values, both sets of values being too high.

The solid fatty acids of liver lecithin are essentially those of the fat stores but in a different proportion, the proportion as calculated

from the molecular weight being 30 for palmitic and 70 for stearic while in the fat of the stores and in the neutral fat of the liver palmitic is found to predominate. Of the liquid fatty acids oleic and linoleic are found in the stored fat and food fat, but ordinarily the proportion of linoleic is very much less than is found in the present work in liver lecithin. Arachidonic acid and other highly unsaturated long chain acids are found only in traces in stored fat and in slightly larger amount in the neutral fat of liver. The presence of the large proportion of linoleic acid, an 18-carbon acid, would fit in with Leathes' hypothesis of desaturation by the liver, originating either from oleic or from the stearic acid. As was noted by Klenk and Schoenebeck (2) the arachidonic acid and the clupanodonic acid ( $C_{22}$ ) found by them in liver do not easily fit into Leathes' scheme of desaturation because in these cases there is an increase in the length of chain. The preponderance of stearic over palmitic in the lecithin acids might be another example of lengthening of the chain by the liver.

The absence of linolenic acid in this fatty acid mixture and apparently in most animal lipids, as has been commented on several times in reports from this laboratory, is worthy of note as compared with its wide distribution in plant fats. When fed in not too large amount it is largely destroyed (Ellis *et al.*) and the presumption is that if formed in the animal organism it is promptly destroyed. However, it is not difficult to get it deposited in the depot fat as Rosenfeld showed long ago. Whether it can be introduced into the phospholipid molecule has not been shown.

The questions of whether the fatty acids in the phospholipids are there as the result of introduction bodily from the transported fatty acids or whether their nature can be changed *in situ* are unanswered. It appears certain that during fat absorption some of the phospholipids of the intestinal mucosa of the liver and blood plasma do undergo these substitutions and it seems likely that all phospholipids may do so. Sinclair has found definitely (25) that the nature of the fatty acids in tissue phospholipids may be changed by food fat and the time relations of the change and the fact that relatively small amounts of certain fats bring about the change strongly suggest a selection of the more highly unsaturated acids by the phospholipids. The factor of selection was mentioned in a previous discussion of Leathes' hypothesis of

fatty acid desaturation by the liver, in connection with the neutral fat of liver (34). However, it is unlikely that selection could account for the large proportion of arachidonic acid found in liver phospholipid. There appears to be no explanation except that of formation on the spot. In this connection the fact was pointed out by André (35) that it is usual for naturally occurring fatty acids to have in their molecule one group of 9 carbon atoms (generally at the carboxyl end) free from double bonds, while the first bond occurs in position  $\Delta^{9-10}$  and the others are spaced so that double bonds come at every 3rd carbon atom. Because of this peculiarity an 18-carbon acid would have a maximum of three double bonds and when four or more bonds are present the chain is longer by 2 carbon atoms for each extra double bond. Thus the commonly occurring fatty acids with the higher degree of unsaturation, arachidonic, etc., are 20- or more carbon acids.

The lengthening of the chain proceeds according to the characteristic rule for changing the length of the chain of the fatty acids, 2 carbon atoms at a time and presumably involves the same hypothetical 2-carbon fragment as is formed when the fatty acids are broken down by  $\beta$  oxidation, but since the extra double bond appears at the end opposite the carboxyl group the change can hardly be a reversal of the  $\beta$  oxidation unless it is assumed that the whole molecule is rearranged.

#### SUMMARY

In the lecithin of beef liver the proportion of liquid (unsaturated) to solid (saturated) fatty acids was found to be about 55:40. The saturated acid of lecithin consisted of about 71 parts of stearic to 29 parts of palmitic acid as compared with the neutral fat of liver which contains more palmitic acid than stearic acid.

The distribution of the unsaturated acids was found to be linoleic 45 per cent, arachidonic 31 per cent, and oleic 21 per cent. Acids with three double bonds were not found. Acids of larger molecule than  $C_{20}$  were present in small amount in one series.

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## CHARACTERISTICS OF OXIDATION BY AZOTOBACTER

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The oxidation of carbohydrates by molecular oxygen at ordinary temperatures has been widely studied. Evans (1) and others studied the course of the oxidation when copper acetate in acid solution was catalyst. Spoehr and Smith (2), Degering and Upson (3), and others studied the catalysis by an iron-pyrophosphate complex in a solution buffered with  $\text{Na}_2\text{HPO}_4$ . Oxidation by  $\text{KMnO}_4$  in alkaline solution and by  $\text{K}_2\text{Cr}_2\text{O}_7$  in acid solution has been studied by several investigators with regard to both extent of completion and intermediate compound formation. Witzemann (4) using  $\text{KMnO}_4$  showed specifically in the case of lactate oxidation that the course of the oxidation is significantly influenced by the experimental conditions and that it proceeds simultaneously by several paths. Kendall, Friedemann, and Ishikawa (5) have studied the chemical activity of *Bacillus coli*, *Bacillus alcaligenes*, *Bacillus pyogenes faetidus*, *Vibrio* H/61, *Staphylococcus aureus*, and *Bacillus pyocyaneus*, and find significant differences in their fermentative and oxidative character. The oxidation of a number of compounds was also studied in connection with work on *Bacillus coli* by Cook and Stephenson (6) and Cook and Haldane (7). Neuberg has studied in detail the course of biochemical oxidations but has in general carried these out in the absence of molecular oxygen. Warburg (8) succeeded in preparing an iron-charcoal model which imitates *in vitro* the oxidation of biochemical compounds, by molecular oxygen, occurring *in vivo*.

[*Azotobacter* possesses certain advantages for studying biochemical oxidations and also nitrogen fixation at ordinary temperatures. As will become evident, the oxidation mechanism involved is

relatively less complex, compared to that of most organisms, especially since *Azotobacter* appears to possess no fermentative or glycolytic metabolism. The energy of metabolism is very probably concerned in the mechanism of nitrogen fixation and general information regarding the physiology of the cell, the nature of its oxidases and other enzymes is of importance in the study of nitrogen fixation. The technique is facilitated by an extremely high rate of oxidation per unit of dry weight. Meyerhof and Burk (9) and others have utilized the rate of oxygen consumption of *Azotobacter* as a measure of growth and nitrogen fixation.

The present investigation has been concerned with the degree of completion to which various substrates are oxidized (chiefly glucose and its fermentation products), the course of the oxidation as indicated by the formation of intermediates, the comparative rates with mixed substrates, and the action of specific and indifferent narcotics on the oxidation enzymes. *Azotobacter vinelandii* has been used exclusively in this investigation and will hereafter be referred to as *Azotobacter*.

### Method

The methods of culture and the Warburg technique used to measure the rates of oxygen consumption (respiration) have been described previously in detail (Burk (10), Burk and Lineweaver (11), and Burk and Milner (12)). The cultures for use in the Warburg vessels have been grown in an air thermostat at 28° for 24 to 48 hours, in sterilized, aerated, 250 cc. gas wash bottles containing 100 cc. of culture medium. The culture medium consisted of 1 per cent glucose dissolved in the clear liquid obtained after the following mixture had been thoroughly shaken, allowed to stand, and settle: 0.8 gm. of  $K_2HPO_4$ , 0.2 gm. of  $KH_2PO_4$ , 0.2 gm. of NaCl, 0.2 gm. of  $MgSO_4 \cdot 7H_2O$ , 0.1 gm. of  $CaSO_4 \cdot 2H_2O$ , 0.01 gm. of  $Fe_2(SO_4) \cdot 9H_2O$ , 1000 gm. of  $H_2O$ .

The organisms obtained after 24 to 48 hours growth were centrifuged and washed twice with culture medium without glucose, suspended in approximately one-half the original volume of culture medium without glucose, shaken vigorously until an even suspension was obtained, and kept at 10°. The stock suspensions varied in dry weight of organisms from 4 to 16 mg. per 100 cc., hence dilution of stock suspensions for use varied with the suspen-

sion used as well as with the type of experiment performed. The activity of the stock suspensions decreased only slightly in 7 days time when kept at 10° (see, *e.g.*, Lineweaver, Burk, and Horner (13)). The respiration of the organisms in the absence of added substrate was practically zero, so that no error occurred from this cause. Unless otherwise stated the pH was maintained at optimum,  $7.1 \pm 0.4$  (Burk and Milner (12), Fig. 8). The various substrates were generally added as follows: 0.2 cc. of a solution whose concentration was 11 times that finally desired was added per 2 cc. of culture.

TABLE I  
*R. Q. and Degree of Oxidation*

	Per cent of complete oxidation	R.Q.	
		Theoretical	Observed
Glucose.....	97.5	1.00	0.985
Lactate.....	105	1.00	1.055
Pyruvate.....	91.5	1.20	1.22
Succinate.....	100	1.14	1.14
Fumarate.....	95	1.33	1.31
Tartrate.....	96	1.60	1.51
Malate.....	93.5	1.33	1.35
Malonate.....	99	1.5	1.6
Acetate.....	95.3	1.00	1.05
Ethyl alcohol.....	104	0.67	$0.67 \pm 0.1$

The respiration quotient was determined according to the method described by Dickens and Simer (14). The CO<sub>2</sub> formed in respiration is absorbed in Ba(OH)<sub>2</sub>, the O<sub>2</sub> consumption being calculated from the observed pressure change. At the termination of the experiment Ba(OH)<sub>2</sub> is tipped from the side-cup into the culture to assure complete absorption of the CO<sub>2</sub>, then excess of 2 N mineral acid is shaken from the well, expelling the CO<sub>2</sub>. The resulting pressure change is used for calculation of the total CO<sub>2</sub> at the end of the experiment. This, corrected for the CO<sub>2</sub> initially present, gives the amount of CO<sub>2</sub> formed in respiration.

Unless otherwise mentioned, the growth attending the oxidation of any substrate was negligible.

*Studies on Complete Oxidation*—The extent of oxidation which a substrate may undergo has been determined by observing what fraction of the oxygen necessary for complete oxidation to carbon dioxide and water is consumed. In a number of cases the R.Q.

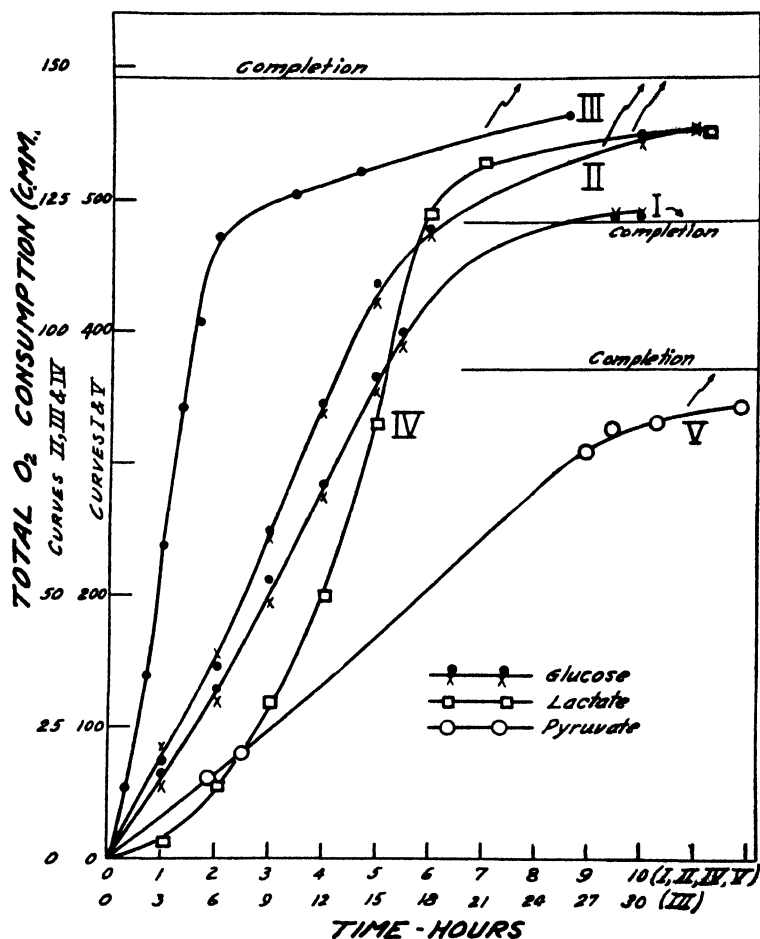


FIG. 1. Oxidation of glucose, lactate, and pyruvate. Curves I, II, and III,  $m/560$ ,  $m/180$ ,  $m/180$  glucose; maximum  $Q_{O_2}$ , 750, 300, 160. Curve IV,  $m/900$  lactate; maximum  $Q_{O_2}$ , 750. Curve V,  $m/300$  pyruvate; maximum respiration rate not comparable to that for lactate and glucose. Lactate and pyruvate points are averages of duplicates. 2 cc. of culture used per vessel.

has also been determined. Although the R.Q. is not quite so accurate a criterion of completion, it has the advantage that the observed value is independent of (1) the initial concentration of

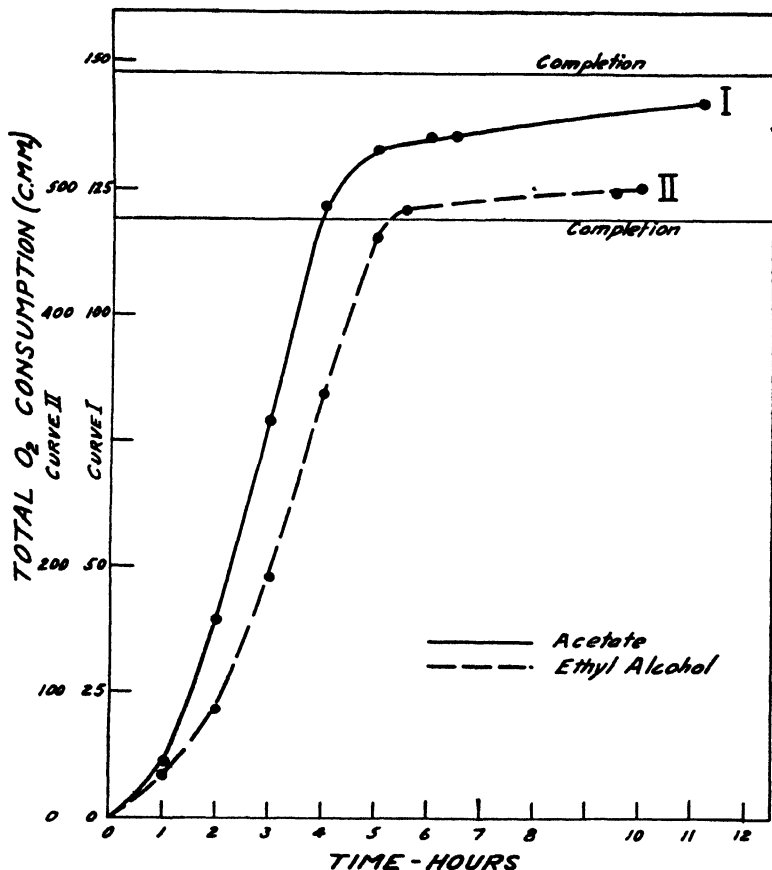


FIG. 2. Oxidation of acetate and ethyl alcohol. Curve I,  $M/600$  acetate; maximum  $Q_{O_2}$ , 800; points averages of duplicates. Curve II,  $M/300$  ethyl alcohol; maximum  $Q_{O_2}$ , 1300. 2 cc. of culture used per vessel.

substrate if oxidation is allowed to go to completion, and (2) any constantly maintained concentration yielding a constant rate of oxygen consumption. The R.Q. is usually accurate to  $\pm 0.03$ .

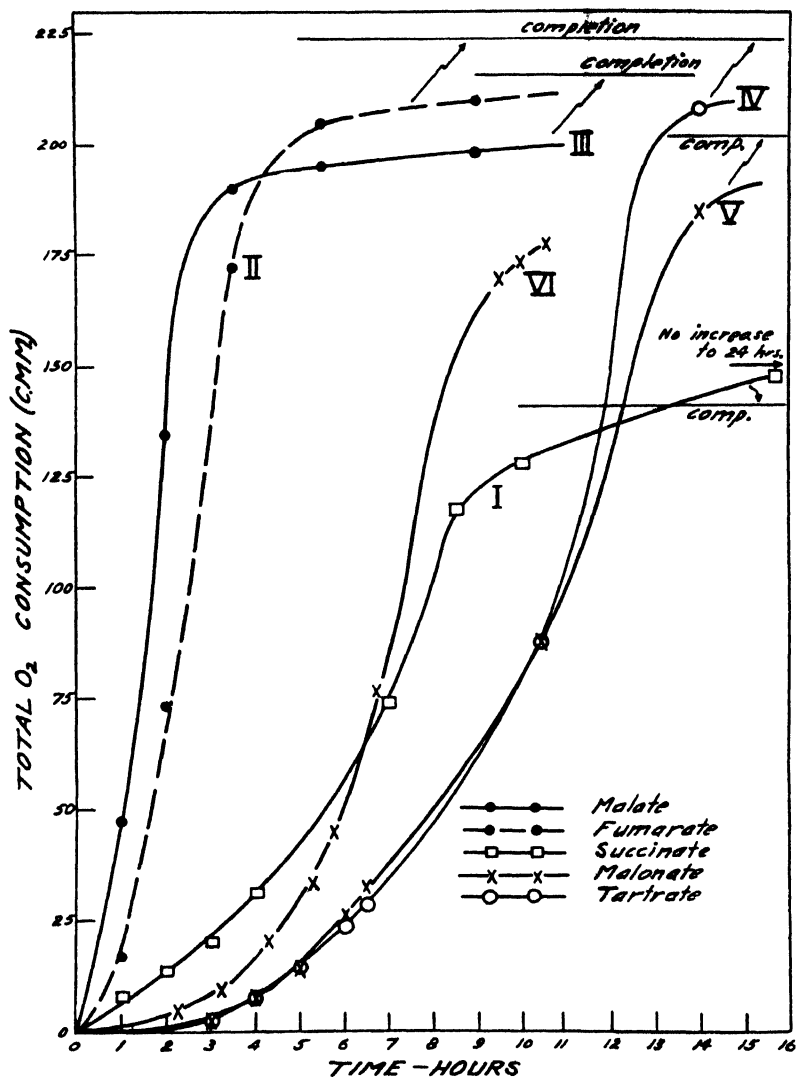


FIG. 3. Oxidation of dicarboxylic acids. Curve I,  $m/1110$  succinate; maximum  $Q_{O_2}$ , 670; rate not comparable to other curves. Curve II,  $m/600$  fumarate; maximum  $Q_{O_2}$ , 400. Curve III,  $m/622$  malate; maximum  $Q_{O_2}$ , 570. Curve IV,  $m/505$  tartrate; maximum  $Q_{O_2}$ , 300. Curves V and VI,  $m/400$  malonate, two different preparations of washed cells; maximum  $Q_{O_2}$ , 230 and 1200. 2 cc. of culture used per vessel.

The oxidation of certain compounds corresponds closely to theoretical completion as indicated both by direct measurement of the percentage oxidation and by R.Q. determinations (Table I).

The  $Q_{O_2}$ , as well as the kinetics of the oxidation is indicated in

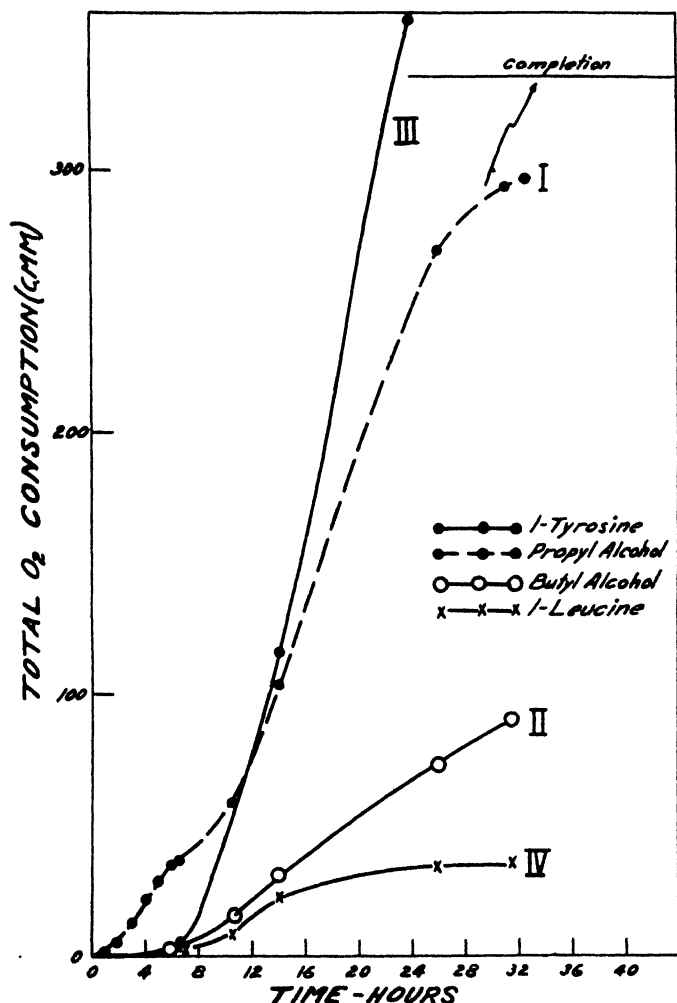


FIG. 4. Oxidation of alcohols and amino acids. Curve I,  $m/600$  propyl alcohol; maximum  $Q_{O_2}$ , 150. Curve II,  $m/600$  butyl alcohol; maximum  $Q_{O_2}$ , 45. Curve III,  $m/600$  *l*-tyrosine; maximum  $Q_{O_2}$ , 230. Curve IV,  $m/600$  *l*-leucine; maximum  $Q_{O_2}$ , 25. 2 cc. of culture used per vessel.

Fig. 1 (glucose, lactate, and pyruvate), Fig. 2 (acetate and ethyl alcohol), Fig. 3 (malate, fumarate, succinate, malonate, and tartrate). The manner in which these curves approach their theoretical limit is also further evidence for the occurrence of complete oxidation. The complete oxidation of pyruvate has been established on the following additional grounds. Commercial pyruvic acid which is partially polymerized gave 30 per cent less oxygen consumption than that further purified by fresh distillation at 10 mm. pressure. A threshold concentration below which the pyruvic acid remains unoxidized is not involved as shown by a constant percentage oxidation with varying initial concentration of acid (unreported data).

The oxidation of certain substrates, propyl and butyl alcohols, and *l*-tyrosine (Fig. 4) and formaldehyde, proceeded at such a slow rate that completion was not observed: The alcohols would almost certainly be expected to go to completion. The observed oxidation of *l*-tyrosine was slightly more than 60 per cent. Formaldehyde at an initial concentration of  $3.7 \times 10^{-4}$  M was observed to give only 66 per cent of the theoretical oxygen consumption after 42 hours, although at least 98 per cent removal from the culture was shown by the fuchsin test. It is uncertain whether 100 per cent oxidation would be attained even with longer periods of time. The study of  $\text{CH}_2\text{O}$  oxidation requires working with 5 to 15 cc. of culture per vessel, since  $\text{CH}_2\text{O}$  inhibits glucose oxidation 50 per cent at  $8 \times 10^{-4}$  M, a concentration corresponding at complete oxidation to only 18 c.mm. of  $\text{O}_2$  per cc. of culture.

Certain substrates were not oxidized at all or only extremely slowly. Acetaldehyde, oxalate, formate, methyl alcohol, maleate, glycollate, and *l*-cystine gave less than 4 per cent oxidation during observations which varied from 9 to 48 hours (5 hours in the case of oxalate). Several concentrations, in general varying from 0.001 to 0.1 M, were employed for each compound some of which, in each case, were below the concentration which would inhibit glucose oxidation 50 per cent. *l*-Leucine (Fig. 4) and cysteine gave about 4 to 5 per cent oxidation in 14 hours. 50 per cent inhibition of glucose oxidation was observed with 0.19 M maleate, 1.3 M methyl alcohol, 0.01 to 0.03 M acetaldehyde, and 0.01 to 0.03 M oxalate. No inhibition of glucose oxidation was observed with 0.1 M formate, 0.1 M glycollate, or 0.0017 M cysteine, *l*-cystine, and

*l*-leucine. In the case of acetaldehyde it was necessary to make observations in the absence as well as in the presence of alkali in order to eliminate condensation in the alkali. An oxygen consumption of 400 c.mm. per 2 cc. of culture at 1 M methyl alcohol (corresponding at completion to 67,200 c.mm. of O<sub>2</sub> per 2 cc.) was found to be due entirely to impurities. It is probable that the apparent slight oxidation of some of these compounds is also due to impurities.

Although many biochemical oxidations result in incompletely oxidized end-products (e.g., *Bacillus coli* oxidizes glucose and lactate stoichiometrically only to two-thirds completion) the general statement seems warranted in the case of *Azotobacter* that if a substrate is oxidized at all it is oxidized completely to CO<sub>2</sub> and water. Side reactions resulting in incompletely oxidized products take place only to a limited extent, if at all. Oxygen consumption, if it occurs, commences within at least 12 hours after addition of the substrate to the organisms. The list of compounds given in this study as oxidizable by *Azotobacter* in liquid culture could undoubtedly be extended very considerably. This is especially true since the cultures used had been previously grown with glucose as the only substrate. Stephenson and Stickland (15) have just shown that a bacterial enzyme may be adaptive; i.e., that it will occur only when the test substrate has been present in the culture during the previous growth.

*Intermediate Metabolism*.—In the oxidation of certain alcohols the occurrence of intermediates has been demonstrated by an increased acidity, and in the oxidation of malonate by the preliminary splitting off of CO<sub>2</sub>.

Ethyl alcohol at 0.04 to 0.1 per cent causes the pH to fall to as low as 5.0 in 4 to 6 hours (1 per cent glucose was present in these experiments). Similar pH lowering also occurs with propyl and butyl alcohols. The intermediate formed during ethyl alcohol oxidation is very probably acetic acid, and not a higher acid; the formation of glycollic or formic acid is entirely eliminated since neither is oxidized and the oxidation of ethyl alcohol is complete.

A marked lag phase occurs in the oxidation of tartrate and malonate as compared to malate and fumarate (Fig. 3). While this lag might be partially due to the formation of an enzyme which is used in the oxidation of the acid it is evident from Table

II that the oxidation takes place chiefly according to the following two steps.



The theoretical R.Q. for the first step is  $\infty$ , for the second step, 1.0, and for the over-all reaction when completed, 1.5. Occurrence of the only other possible intermediate,  $\text{CH}_4$ , is precluded by the absence of positive pressure in the vessels containing alkali. The start of, and increase in the rate of,  $\text{O}_2$  consumption is due to the formation and building up of the acetate concentration. These studies add carboxylase to the list of enzymes previously demonstrated in *Azotobacter* (*viz.*, catalase (Bonazzi (16) and Burk,

TABLE II  
*Oxidation of Malonate*

Time	$\text{CO}_2$	R.Q.
<i>hrs.</i>	<i>c.mm.</i>	
1	6	$\infty$
$3\frac{1}{4}$	52	4.7
$5\frac{1}{4}$	118	3.9
23	284	1.6

Horner, and Lineweaver (17)), oxidase, and those involved in nitrogen fixation).

Explanations based on intermediates could doubtless be experimentally confirmed in a similar manner for the course of the oxidation in several other cases. The tartrate oxidation curve compares almost identically with that of malonate; fumarate gives a curve similar to that of malonate but with a much shorter lag phase.

*Comparative Rates of Oxidation of Different and Mixed Substrates*—Comparison of rates of oxidation when various substrates are oxidized alone and in mixture yield information concerning the specificity of the oxidative enzymes and the course of oxidation. Columns 1 to 3, Table III, compare the rates of oxygen consumption of washed cells at  $31^\circ$  with glucose, succinate, and lactate near their

optimum concentrations. The  $Q_{O_2}$  values (c.mm. of  $O_2$  consumed per mg. of dry weight per hour) are very much larger than those found for yeast or mammalian tissue, where  $Q_{O_2}$  values of 60 and 20, respectively, are normal, or *Bacillus coli* where  $Q_{O_2}$  for various substrates varies from 0.48 to 3.3 (Cook and Haldane (7)).

The fact that the rates with mixed substrates are less than the sums of the independent rates (Columns 4 to 9), indicates one of three possible oxidation mechanisms: (1) related enzyme systems in which each enzyme system is partially inhibited by the presence of the other substrate; (2) competition for a single enzyme; or (3) intermediates formed are oxidized by a common enzyme. In any

TABLE III  
*Comparison of Rates with Mixed Substrates*

	C.mm. O <sub>2</sub> consumption per 3 cc. per half hr.								Per cent of sum of rates attained by mixture			
	1 per cent glucose	0.7 per cent succinate	0.7 per cent lactate	Glucose and succinate		Glucose and lactate		Succinate and lactate		(4) and (5)	(6) and (7)	(8) and (9)
				Sum	Mixture	Sum	Mixture	Sum	Mixture			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
By 1st hr.....	21	64	30	85	59	51	39	94	34	69	77	36
“ 3rd “ .....	48	86	67	134	100	115	89	153	72	75	77	47
“ 5th “ .....	58	88	98	146	110	156	110	186	105	75	71	56
Q <sub>O<sub>2</sub></sub> , 5th hr.....	1290	1960	2180		2440		2440		2340			

of these mechanisms additive rates could be obtained only at relatively low concentrations. The data eliminate the possibility of the occurrence of two enzyme systems, each of which acts independently of the other's substrate.

*Dehydrogenase Enzymes*—During the process of respiration hydrogen might be removed from the organic substrates in such a manner that it need not be transferred to molecular oxygen; that is, dehydrogenation might take place by an enzyme system capable of acting independently of the oxygen-activating system. Such enzymes, designated as dehydrogenases, occur in most organisms and are customarily tested for by the methylene blue technique (Stephenson (18) p. 36).

*Azotobacter* washed free of glucose was suspended in the presence of 0.5 per cent of certain substrates and placed in an atmosphere of nitrogen. After 6 hours the following percentage reductions of 10 parts per million of methylene blue were observed: glucose 98 per cent; lactate and pyruvate 80 per cent; succinate, malate, tartrate, and malonate 70 per cent; fumarate 0 per cent; controls with no substrate 0 per cent; controls with glucose but no culture 0 per cent. 100 per cent reduction occurred within 10 hours in all cases except the fumarate and the controls.

Keilin (19) has attributed the respiration inhibition produced by HCN to inhibition of the oxygen-activating mechanism, and that produced by urethane to inhibition of the reducing mechanism of the cells, *viz.* the dehydrogenases.

Glucose dehydrogenase of *Azotobacter* is not inhibited even at  $2.2 \times 10^{-3}$  M HCN whereas 50 per cent inhibition of respiration occurs at  $5 \times 10^{-6}$  M HCN. Stimulation was observed at the higher concentrations of HCN. This is typical of certain dehydrogenase systems in other organisms. 50 per cent inhibition of dehydrogenase activity by urethane occurs between the concentrations 0.16 and 0.26 M compared to the 0.15 M concentration required for 50 per cent inhibition of respiration. Thus it is evident that *Azotobacter* possesses dehydrogenases which behave in a typical manner with respect to HCN and urethane.

*Respiration Inhibition by Specific Narcotics*—According to Warburg the respiration enzyme (*Atmungsferment*) has an essential heavy metal constituent, normally iron. The inhibition caused by HCN, H<sub>2</sub>S, and Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> supports this view in the case of *Azotobacter*.

Meyerhof and Burk (9) have reported 50 per cent inhibition of respiration by  $5 \times 10^{-6}$  M HCN. Experiments by the writer on young active cultures confirm this figure. 100 per cent inhibition was observed at  $10^{-4}$  M but the minimum concentration for 100 per cent inhibition is probably less than  $4 \times 10^{-5}$  M. H<sub>2</sub>S under similar conditions inhibits respiration 50 per cent at about  $2$  to  $3 \times 10^{-4}$  M. Although these figures indicate that a heavy metal-containing enzyme is responsible for oxygen activation in the sense of Warburg, the possibility that the oxidized substance as well as the oxygen must be activated is not affected. Such a mechanism is probable in the case of *Azotobacter* (Burk (20)),

and also in the case of lactate dehydrogenase prepared by Stephenson (21).

*Hecht-Eicholtz Reagents*—According to Hecht and Eicholtz (22), Eicholtz (23), and others, certain organic compounds may be

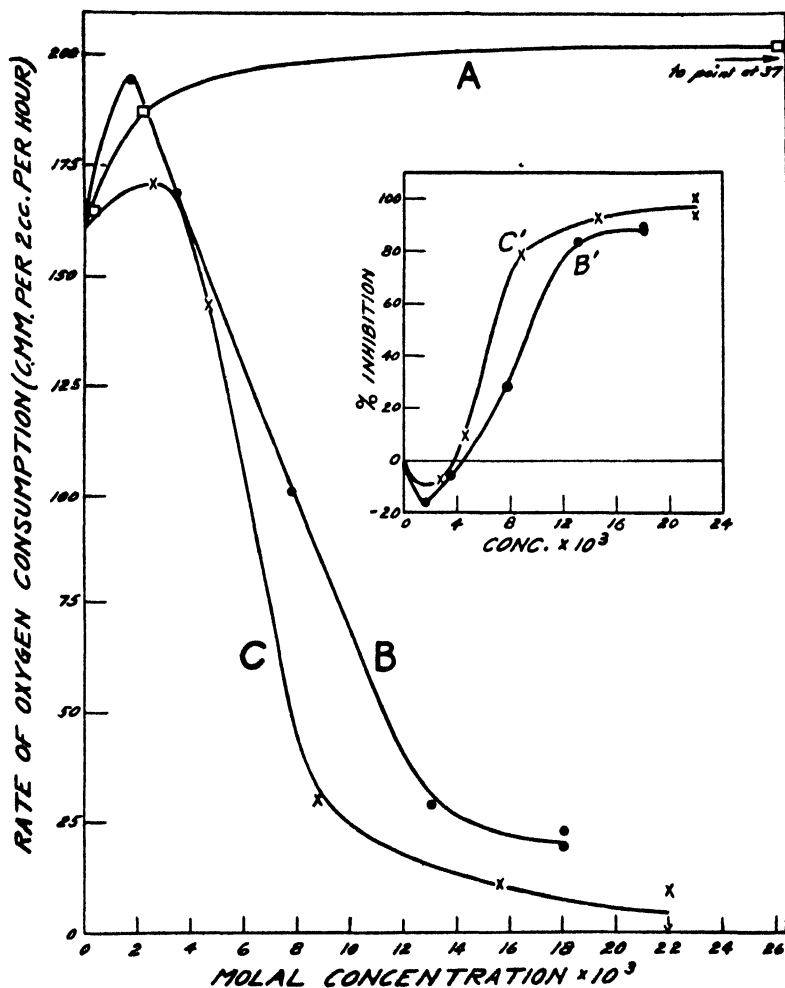


FIG. 5. Inhibition by Hecht-Eicholtz reagents. Curves A, B, and C correspond to the compounds given in the text. Curves B' and C' are replots of Curves B and C. 2 cc. of 2 day unwashed culture used; glucose about 0.5 per cent.

used as heavy metal reagents in biochemical research. The following compounds have been studied in the case of *Azotobacter*. Reagent A, *o*-aminophenol-*p*-sulfonic acid, which forms a complex with Fe; Reagent B, 1-amino-8-naphthol-4-sulfonic acid, which forms complexes with Cu and Fe; and Reagent C, 8-hydroxy-quinolinesulfonic acid, which forms complexes with Cu, Mn, Co, and Ni.

TABLE IV  
*Respiration Inhibition by Homologous Series*

Compound (1)	Con- centra- tion (2)	Inhibi- tion (3)	Concentration yielding approximately 50 per cent inhibition		Ratio	
			Warburg (4)	Meyer- hof (5)	(2)/(4) (6)	(2)/(5) (7)
	<i>M</i>	<i>per cent</i>	<i>M</i>	<i>M</i>		
Methyl alcohol	1 3	40-60	5 0	0 025	0 26	53
Ethyl "	0 75	45-55	1 6	0 08	0 48	9 1
Propyl "	0 25	45-55	0 8	0 016	0 31	16
Butyl "	0 060	40-60	0 15		0 4	
Amyl "	0 032	40-60		0 0046		7 1
Heptyl "	0 004	30-70		0 0004		10
Octyl "	0 001	30-70				
Urea	0 4	40-60		0 047		8 3
Methylurea	0 24	45-55	0 6 1 4		0 24	
Phenylurea	0 008	30-70	0 023	0 0029	0 35	2 5
Urethane	0 15	45-55				
Methylurethane	0 1	30-70	0 7-1 3	0 033	0 1	3.0
Ethylurethane.	0 09	45-55	0 4	0 020	0 23	5 0
Phenylurethane	0 005	40-60	0 003-0 006	0 0013	1 1	3 3

1 and 2 day cultures were used in all cases, with respirations of 40 to 50 c mm. of O<sub>2</sub> per cc. per hour.

It is seen in Fig. 5 that Curves B and C, obtained with Reagents B and C respectively, inhibit respiration 50 per cent at  $9 \times 10^{-3}$  M and  $7 \times 10^{-3}$  M, respectively. These compounds both combine with Cu. Reagent A, which does not inhibit respiration even at  $3.7 \times 10^{-2}$  M, combines with Fe, but not with Cu. While this indicates, on the basis of the work of Hecht and Eicholtz, that

## H. Lineweaver

Cu is an integral part of an enzyme or enzymes concerned in *Azotobacter* respiration, certain unconfirming features are present. It was found that Reagent C prevented Cu inhibition but that no reversing mass action effect, restoring respiration, is exerted by the addition of small amounts of Cu to cultures inhibited by Reagent C. Furthermore the concentrations of Reagents B and C required to produce 50 per cent inhibition approach the order

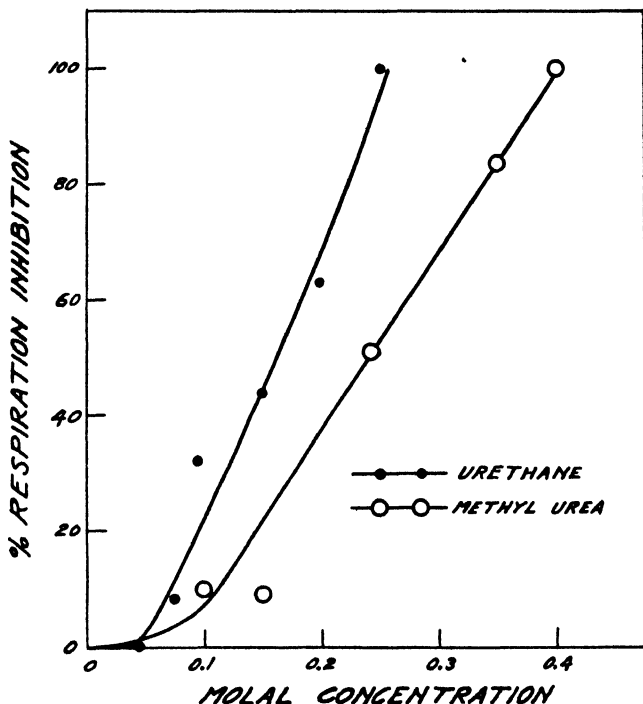


FIG. 6. Inhibition by homologous series narcotics

of concentration for the indifferent homologous series narcotics (see Table IV, phenylurea, phenylurethane, and the higher alcohols). Curve B' does not approach 100 per cent inhibition because slight autoxidation takes place at the higher concentrations under the conditions employed. Reagents A and C, however, were not appreciably oxidized.

*Respiration Inhibition by Homologous Series of Indifferent*

*Narcotics*—Table IV gives the molal concentrations of the homologous series narcotics, alcohol, urea, and urethane which produce 50 per cent inhibition of respiration. These concentrations have been obtained from plots of data in general similar to that illustrated in Fig. 6. The probable limits of accuracy for each compound are indicated by the figures in Column 3. In Column 4 are given the

TABLE V  
*Respiration Rate Inhibition As Function of Age of Culture*

Age, days.....	1	2	3	4	5	6	7	9	11	13	22
Experiment 1											
Rate of respiration, <i>c.mm. O<sub>2</sub></i> <i>per cc. per hr.</i> .....	30	35	103				46		52		
Inhibition, 0.7 M ethyl alcohol, <i>per cent</i> .....	29	27	35				32		33		
Experiment 2											
Rate of respiration, <i>c.mm. O<sub>2</sub></i> <i>per cc. per hr.</i> .....	12	53		74	99	43			62	83	41*
Inhibition, 0.125 M methylurethane, <i>per cent</i> .....	67	74		85	80	79			79	80	65
	(½ day)										
Experiment 3											
Rate of respiration, <i>c.mm. O<sub>2</sub></i> <i>per cc. per hr.</i> .....	15		103					80			
Inhibition, <i>per cent</i>											
0.24 M urethane.....	50							60			
0.3 " propyl alcohol.....	52		54					59			
0.2 " " " .....			32					38			

Experiment 1, pH  $6.8 \pm 0.1$  (air); Experiment 2, pH 6.0 to 6.8 (21 per cent O<sub>2</sub> in H<sub>2</sub>); Experiment 3, pH  $6.7 \pm 0.2$  (air).

\* 1 per cent glucose added 3 hours before measurements, in this very old culture.

molal concentrations which, according to Warburg, yield an inhibition of respiration between 30 and 70 per cent, in various cells, red blood cells of birds, vibrios, liver cells, and the central nervous system of frogs. In Column 5 are given the molal concentrations which according to Meyerhof (24) yield approximately 50 per cent inhibition of respiration in nitrifying bacteria. Columns

6 and 7 compare the 50 per cent inhibition concentrations for *Azotobacter* with those obtained by Warburg and Meyerhof. In general, *Azotobacter* is two to three times as sensitive toward these narcotics as the cells studied by Warburg, but only one-quarter to one-tenth as sensitive as the nitrifying bacteria studied by Meyerhof. Although the inhibition function for a series of narcotics is different for different species of organisms, it is essentially constant for a given organism.

TABLE VI  
*Reversibility of Respiration Inhibition by Indifferent Narcotics*

Narcotic	Propyl alcohol	Butyl alcohol	Control		
A. Volume of culture, 1 cc.					
Concentration, <i>M</i>	0 00	0 22	0 00	0 05	
Rate of respiration, <i>c mm. O<sub>2</sub> per vessel per hr.</i>	46 5	26 9 25 7	46 5	38 4 37 4	46 5
Inhibition, <i>per cent</i>		43 5		19 5	
B After addition of 3 cc. of inorganic medium					
Concentration, <i>M</i>	0 055*	0 055	0 0125*	0 0125	
Rate of respiration, <i>c mm O<sub>2</sub> per vessel per hr</i>	45 2	48 3	60 5	54 7	56.3
Inhibition, <i>per cent</i>	20 0	16 2		3 0	

A 1 day culture was used. Section B compares the normal inhibition obtained (second and fourth columns) with that obtained when the concentration of the narcotic in Section A was reduced to one-fourth its original concentration by dilution.

\* Appropriate amounts of narcotic were added.

Table V shows that the inhibition is not a function of the age of the culture even though other characteristics of *Azotobacter* change quite markedly with age; *viz.*, the morphology, growth velocity, and *Q<sub>0</sub>*. This fact is equally explainable on the basis of either indifferent adsorption or chemical reaction. Evidence, however, is in favor of the occurrence of inhibition by indifferent (general) adsorption on a surface or surfaces of the cell. Warburg (8) has shown that the increase in inhibition for a given concentration of several members of a homologous series is a direct function of the respective adsorption coefficients.

Table VI shows, in further support of the adsorption theory of inhibition, that the effect of the indifferent narcotics, propyl and butyl alcohols, is reversible. The reversibility or the inhibition eliminates chemical reactions which *permanently* remove or transform integral constituents of the respiration enzyme.

#### SUMMARY

1. *Azotobacter vinelandii* is capable of oxidizing, by means of molecular oxygen, a large number of compounds including the following: glucose, lactate, pyruvate, succinate, acetate, malonate, malate, tartrate, fumarate, ethyl, propyl, and butyl alcohols, formaldehyde, and at least one amino acid, *l*-tyrosine.

2. It does not appreciably oxidize formate, acetaldehyde, glycollate, oxalate, cystine, or *l*-cysteine; *l*-leucine is oxidized only very slowly if at all. Maleate, a stereoisomer of fumarate, is not oxidized.

3. The formation of intermediate compounds has been specifically demonstrated in the cases of malonate and ethyl alcohol, the intermediate being acetic acid in both cases.

4. In general, if a substrate is oxidized at all, it is oxidized practically entirely (at least 95 to 100 per cent) to  $\text{CO}_2$  and water.

5. The presence of a malonate carboxylase enzyme has been demonstrated.

6. The presence of typical dehydrogenases has been demonstrated by the methylene blue technique and certain narcotics.

7. The velocity of oxidation of many substrates increases at first, over a period of several hours, before finally attaining a more or less constant value. Either intermediate compound formation or enzyme formation may be involved.

8. The velocity of oxidation with mixed substrates is in general not additive.

9. The  $Q_{10}$  values for the various substrates oxidized was very high compared to that for other organisms. The maximum value for many substrates under ideal conditions varies from 1000 to 4000.

10. The effect of HCN and  $\text{H}_2\text{S}$  indicated heavy metal catalysis of respiration. The Hecht-Eicholtz reagents suggest but do not prove that Cu is involved.

11. The 50 per cent inhibition concentrations for the alcohol,

urethane, and urea homologous series have been reported. The inhibition is not a function of age and in the case of propyl and butyl alcohols is reversible.

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## STUDIES ON ANIMAL DIASTASES

### III. A COMPARISON OF SEVERAL DIFFERENT METHODS FOR THE QUANTITATIVE ESTIMATION OF DIASTASE IN BLOOD

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Within comparatively recent years, many studies have been carried out on blood diastase, with the object in view, first, of elucidating the source, or sources, of this enzyme in the circulating blood, and, secondly, of determining the part, if any, which it plays in carbohydrate metabolism. From a review of the literature, it is evident that the findings of different investigators have in many instances failed to agree. It was thought that this lack of uniformity in the results obtained might be due to differences in the methods used to estimate quantitatively the amount of diastase in the blood, and that a comparison of the most commonly employed methods might throw some light on this subject.

Hitherto, the quantitative estimation of diastase in blood has been effected by one of four different methods: (1) the method of Wohlgemuth (1), which depends on the rate of disappearance of soluble starch, as shown by the starch-iodine color test; (2) the achromic point method of Roberts (2), which is also dependent upon the reaction of the products to iodine; (3) sugar reduction methods, by which the enzyme is quantitatively estimated according to the amount of reducing sugars formed from the hydrolysis of starch or glycogen; (4) the viscosimetric method of Elman and McCaughan (3). This method depends on the time required to effect a 20 per cent reduction in the viscosity of a solution of soluble starch.

Evans (4) in a criticism of the Wohlgemuth and achromic point methods, concludes that, for quantitative comparisons, neither method is satisfactory, and that the best results are obtained with a

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sugar reduction method. He believes that the incubation period should be a short one (10 minutes) and that the strength of the soluble starch solution should be such that less than 30 per cent of it is hydrolyzed, at which stage, the starch-enzyme mixture will still give a blue color with iodine. Sherman and Schlesinger (5) studied the action of amylases in two general ways, by observations, first, on the "amylolytic" power (splitting of starch into products which do not give a blue color with iodine), and, secondly, upon the "saccharogenic" (sugar-forming) power. They show that for different pancreatic amylase preparations, there is a fairly constant relationship between the amylolytic and the saccharogenic powers. They point out, however, that unless the soluble starch solution employed is homogeneous, so that it is fully exposed to the digestive hydrolysis, the value for the amylolytic power is misleadingly low. It is significant that in all subsequent studies, they estimate the saccharogenic, rather than the amylolytic power of these purified amylase preparations.

Elman and McCaughan (3) advocate the use of a viscosimetric method for the quantitative estimation of diastase in blood. In a criticism of the Wohlgemuth method, they state that much difficulty is experienced in selecting an end-point and that the same specimen of blood gives widely divergent values for the diastatic activity. In addition, they contend that, compared to their own method, the sugar reduction methods are more time-consuming, and have often led to conflicting results. In support of their viscosimetric method, they quote Bayliss (6), who believes that in comparing enzyme solutions of different strengths, the time taken to effect an equal change in the reactants best serves as a basis of comparison, especially, if, as in starch hydrolysis, the reaction takes place in steps.

In the present investigation, the Wohlgemuth, the viscosimetric, and several sugar reduction methods were compared, in an attempt to decide which is the most satisfactory for the quantitative estimation of diastase in blood.

#### EXPERIMENTAL

A comparison of the starch-iodine method of Wohlgemuth (1), the viscosimetric method<sup>1</sup> of Elman and McCaughan (3), and the

<sup>1</sup> We are indebted to Dr. D. Roy McCullagh of the Cleveland Clinic for the use of his viscosimetry apparatus.

sugar reduction method of Myers and Killian (7) was made. A series of eleven bloods, widely differing in diastatic activity, was selected and the serum separated off. It has been stated (3) that if placed in the refrigerator, blood serum can be kept for 24 hours without suffering any loss in diastatic activity. It was thought best, however, to carry out all estimations on fresh blood, and in all our experiments our determinations were made within an hour of drawing the blood samples. The soluble starch, em-

TABLE I  
*Diastatic Activity\* of Blood by Different Methods*

Blood serum used	Myers-Killian method	Diastatic activity found			
		Wohlgemuth method		Viscosimetric method	Myers-Killian method Viscosimetric method
		D†	Time at 40°		
			min.	units	
Normal dog	33.8 per 0.2 cc. serum	13.3	30	15.8	33.8/15.8 = 2.14
	27.9 " 0.2 " "	10.0	30	14.6	27.9/14.6 = 1.91
	35.3 " 0.2 " "	13.3	30	18.5	35.3/18.5 = 1.91
	40.1 " 0.2 " "	13.3	30	19.4	40.1/19.4 = 2.06
Depancre- atized dog	21.4 " 0.2 " "	10.0	30	10.4	21.4/10.4 = 2.05
	24.5 " 0.2 " "	8.9	30 hrs.	12.7	24.5/12.7 = 1.93
Normal human	38.9 " 2 " "	16.0	2	1.88	3.89/1.88 = 2.12
	28.6 " 2 " "	8.9	2	1.44	2.86/1.44 = 1.98
Diabetic human	45.1 " 2 " "	16.0	2	2.12	4.51/2.12 = 2.12
	37.7 " 2 " "	13.3	2	1.93	3.77/1.93 = 1.95
	47.6 " 2 " "	20.0	2	2.47	4.76/2.47 = 1.90

\* As our index or unit in diastase estimations in blood we have employed the per cent of starch (10 mg.) converted to reducing sugar by the amount of blood used (2 cc. for human, 0.2 cc. for dog).

† The symbol, *D*, employed by Wohlgemuth refers to digestion at a definite time and temperature.

ployed throughout our work, was prepared from potatoes by the method of Small (8).

It will be seen from Table I that there is a fairly close relationship between the values for the diastatic activity obtained by the viscosimetric method and the sugar reduction method of Myers and Killian. It would seem, then, that, although according to Elman and McCaughan, theoretically, the viscosimetric method

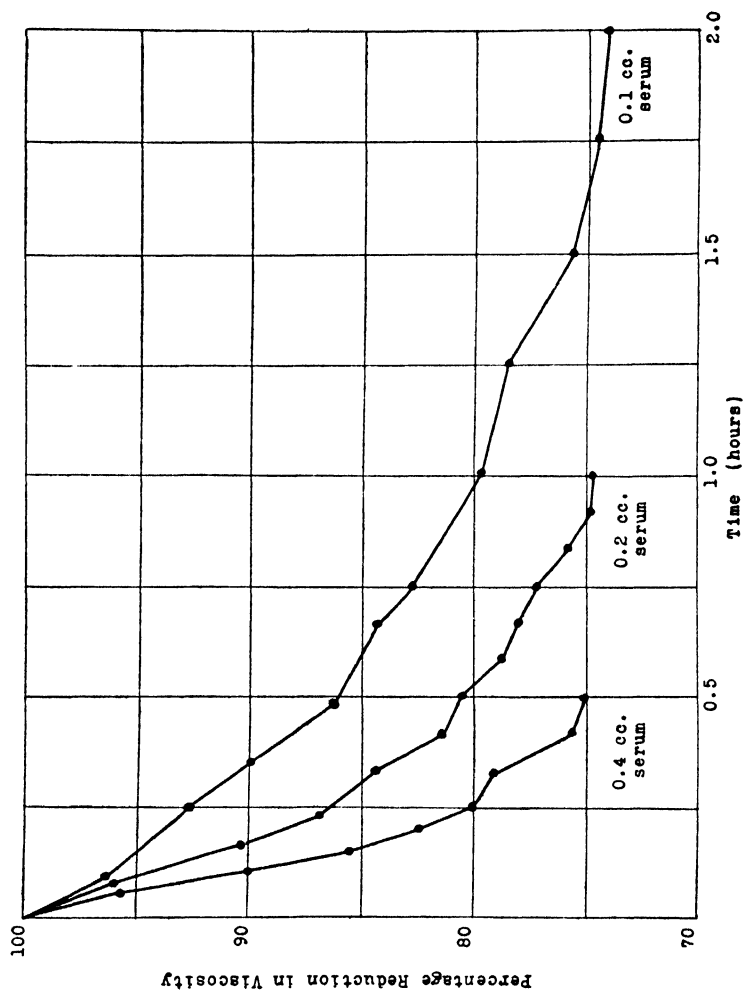


FIG. 1. Viscosimetric method. Reduction in viscosity of starch with time with different amounts of normal dog serum.

is the more accurate, from a practical standpoint either of the two methods is satisfactory. Since in the viscosimetric method the blood-starch mixture is buffered, and in the Myers-Killian method it is not, and since the values for the diastatic activity are closely proportional by both methods, buffering of the blood-starch mixture would appear to be unnecessary. With regard to the Wohlgemuth method, the same proportionality is not observed. Our experience with this method is that an accuracy of  $\pm 25$  per cent only, can be expected. We are of the opinion that it is unsuitable for the quantitative estimation of diastase, especially if it is desired to detect only small changes in the enzyme content of blood.

In Fig. 1 is shown the drop in viscosity of a 3 per cent starch solution when different amounts of normal dog serum are added. It will be noted that the time required to effect an equal change in viscosity is inversely proportional to the amount of serum added to the starch, except in the very early stages of the reaction. These findings are in close agreement with those of Elman and McCaughan. The amount of change arbitrarily selected by these authors, a 20 per cent reduction in viscosity, would appear, then, to be completely satisfactory.

Although our findings show that there is a fairly close relationship between the amount of serum used and the time required to effect a 20 per cent drop in the viscosity of the soluble starch solution, when the diastase content is very low, considerably more than 0.4 cc. of serum has to be added to 5 cc. of starch solution in order to produce a 20 per cent drop in the viscosity within a reasonable time. If this should be the case, a correction for the difference between the viscosity of the starch solution and of the serum would have to be made, and the method, therefore, would lose one big advantage which it has over the other procedures for estimating blood diastase quantitatively; namely, the relatively small amount of blood necessary to carry out a determination. In addition, it is pointed out that the viscosity of the serum added does not change. Hence, if the volume of serum added is relatively large, as it would have to be when the blood diastase is low, another correction would be necessary.

Employing the picric acid method of Myers and Killian (7), we found in this case, also, a close proportionality between the amount

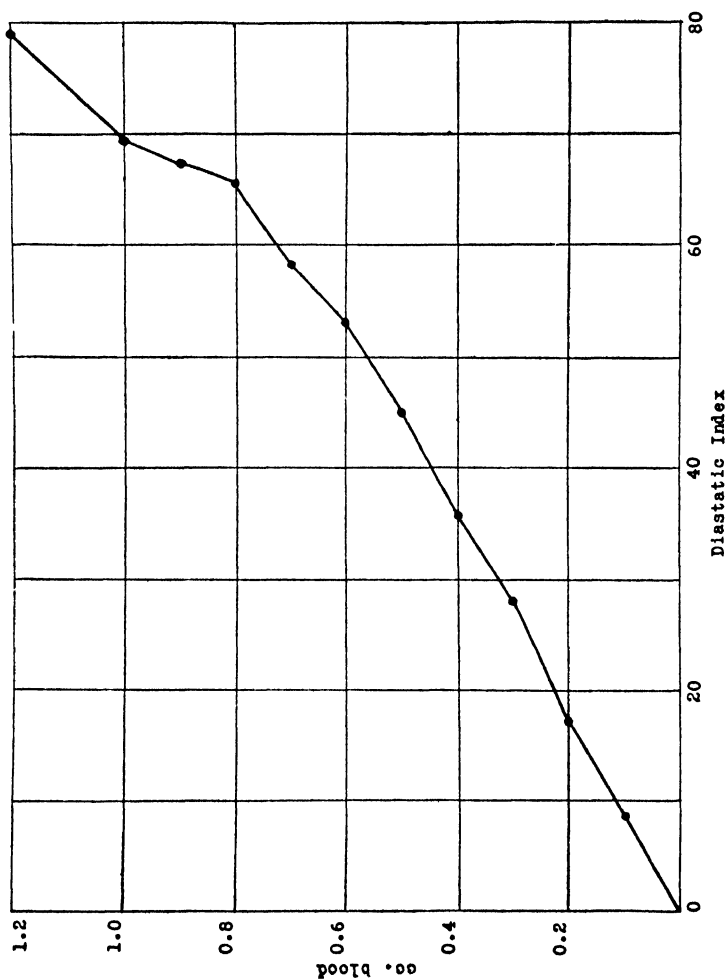


Fig. 2. Myers-Killian method. Showing linear relationship between diastatic index found and amount of blood used.

of blood used and the diastatic activity values, as is shown in Fig. 2. In this experiment, whole blood was employed. It would appear that, up to 60 per cent conversion of the soluble starch to reducing sugars, there is a linear relationship between the blood diastase values and the amount of blood used. Provided, therefore, that sufficient blood to give a diastatic activity value between, say, 10 and 60 is employed, the method is entirely satisfactory. An accuracy of about  $\pm 7$  per cent is easily attainable.

Further investigations were carried out in which several sugar reduction methods were compared. The method devised by

TABLE II  
*Diastatic Activity\* of Dog Blood by Different Sugar Reduction Methods*

Blood used	Myers-Bailey	Hagedorn-Jensen	Folin-Wu	Folin	Benedict	
					With sodium sulfite	Without sodium sulfite
cc.						
0.10	7.8	7.3	3.4	2.8	3.9	3.8
0.20	16.8	15.4	10.8	9.8	8.6	9.6
0.30	25.1	25.3	15.4	16.8	14.2	15.8
0.40	34.0	32.3	21.8	21.4	20.5	23.1
0.50	42.1	37.1	25.0	25.4	25.3	27.5
0.60	50.3	46.0	28.9	30.5	29.8	32.2
0.80	64.8	60.9	41.2	38.9	37.0	42.5
1.00	73.0	70.5	48.9	46.6	48.0	49.4
1.20	77.9	76.7	59.4	56.5	53.5	58.2

\* See note to Table I.

Myers and Killian (7) was employed, except that after incubation at 40° for 15 minutes, the reducing sugars from the starch hydrolysis were estimated according to the methods of Folin and Wu (9), Folin (10), Benedict (11), and Hagedorn and Jensen (12), as well as that of the Myers and Bailey (13) modification of the Lewis and Benedict method. Whole blood, from a normal dog, was used in this experiment.

The results are given in Table II. It will be noted that in all the methods, there is a fairly close proportionality between the amounts of blood used and the diastatic index found, except when values for the diastatic index over 65 or under 8 are encountered.

The picric acid and the Hagedorn and Jensen methods, however, give values for the diastatic activity about 60 per cent higher than the other sugar reduction methods, and for this reason we are of the opinion that the former two are to be preferred to the others.

Ottenstein (14), in her work on blood diastase, uses the Hagedorn-Jensen technique to estimate the reducing sugars. Her method differs from that of Myers and Killian also, in having a 2 hour incubation period and in using a larger amount of soluble starch. The advantage in using this procedure is that blood diastase estimations can be made on finger blood, since only two samples of 0.1 cc. are required.

We have made, on several different subjects, a comparison of the methods of Myers and Killian and of Ottenstein. The results

TABLE III

*Comparison of Myers-Killian and Ottenstein Methods for Diastatic Activity\* of Blood*

Blood used	Myers-Killian 15 min. incubation period	Ottenstein 2 hr. incubation period
Normal human	15.6 per 2 cc. blood 18.5 " 2 " "	57.6 per 2 cc. blood 67.1 " 2 " "
Diabetic human	16.5 " 2 " " 21.3 " 2 " "	62.7 " 2 " " 76.1 " 2 " "
Normal dog	15.6 " 0.2 " " 19.9 " 0.2 " "	59.8 " 0.2 " " 71.1 " 0.2 " "
Deplancreatized dog	17.9 " 0.2 " "	66.3 " 0.2 " "

\* See note to Table I.

by both methods, for purposes of comparison, are expressed as the percentage of soluble starch converted to reducing sugars by 2 cc. of human blood, or by 0.2 cc. of dog blood. It will be seen from our findings (Table III) that the diastatic indices found by the two different procedures are in proportion but that the Ottenstein method gives much lower results. This may be due to the 2 hour incubation period used in the latter procedure. It is our opinion that a short incubation period is essential in sugar reduction methods.

In this belief, an attempt was made to modify Ottenstein's procedure so that a 15 minute incubation period would suffice. It was found, however, that on the same sample of human blood,

widely divergent results were obtained, due to the large experimental error involved in dealing with such small quantities of reducing sugars formed from the starch hydrolysis. With dog blood which has a diastatic activity about 10 times as high as that of humans, results in close agreement with those by the Myers-Killian method were obtained. It is believed, then, that the Ottenstein procedure can only be modified to allow a 15 minute incubation period, for blood (such as dog's) with a high diastatic activity.

In an attempt to explain why the diastatic indices found by the Myers-Bailey and Hagedorn and Jensen methods should be higher

TABLE IV  
*Percentage of Reducing Substances in Soluble Starch, Dextrin, and Maltose  
(Expressed As Glucose)*

Method used.....	Myers-Bailey	Hagedorn-Jensen	Folin-Wu	Folin	Benedict; with sodium sulfite	Benedict; without sodium sulfite
Soluble starch (prepared by Small's method (8)).....	1.42	0.75	0.18	0.23	0.20	0.18
Soluble starch (Kahlbaum).....	14.10	8.96	1.97	3.42	3.11	3.03
“ “ (Lintner).....	4.35	2.48	0.76	1.21	0.70	0.69
Dextrin (Kahlbaum) .....	13.33	10.60	2.96	5.08	4.78	4.72
Maltose (technical) (Chemical Rubber).....	67.90	59.10	33.30	45.70	38.90	37.90
Maltose (pure) (Eastman Kodak)	80.20	75.50	43.30	50.90	46.50	46.70

than those obtained by other sugar reduction methods, the percentage of reducing substances, expressed as glucose, in several soluble starch preparations, dextrin, and maltose, were estimated, all six methods being used. The results are given in Table IV. It will be observed that the Myers-Bailey method gives the highest values, the Hagedorn-Jensen method slightly lower, and the remaining four methods much lower. These results are in agreement with the findings of Pucher and Finch (15) on the reduction values of maltose by different methods. Morrison (16) in the senior author's laboratory also observed this difference in the reducing power of maltose with picric acid and the Folin-Wu reagent. It would seem, therefore, that we have here an explana-

tion of the relatively low values for the diastatic index of blood found when the Folin-Wu, Folin, and Benedict methods are used. When blood diastase acts on soluble starch, the hydrolysis is apparently not carried much, if at all, beyond the maltose stage in the short period of incubation which we used. If we assume that a large proportion of the reducing sugars formed consists of maltose, the low diastatic index found with a sugar reduction method giving a low reduction value for maltose, is readily accounted for. That maltose is formed, we have no doubt, since we have succeeded in forming the osazone. What percentage of reducing sugars consists of maltose has not yet been determined. It may be noted that this finding is not in harmony with the view recently expressed by Somogyi (17) that the sugar formed by the action of blood diastase is a trisaccharide.

#### SUMMARY

A comparison of several different methods for the quantitative estimation of diastase in blood has been made. It has been shown that the most reliable are the viscosimetric method of Elman and McCaughan and the sugar reduction method of Myers and Killian. The former, however, is unsuitable when blood samples low in diastatic activity are to be estimated.

In all sugar reduction methods, there is a linear relationship between the amount of blood used and the diastatic index found. The Myers-Bailey and the Hagedorn-Jensen methods are regarded as being the most satisfactory for estimating the reducing sugars formed from starch digestion, since these methods give the highest reduction values for maltose, of which it is probable the reducing sugars chiefly consist.

When dealing with small laboratory animals, the method of Ottenstein, modified to allow a 15 minute incubation period, is recommended.

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## STUDIES ON ANIMAL DIASTASES

### IV. THE EFFECT OF INSULIN ON THE DIASTATIC ACTIVITY OF THE BLOOD IN DIABETES

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Although in recent years a considerable number of investigations have been carried out on the diastatic activity of the blood in diabetes mellitus, the results obtained by the various investigators are so conflicting that it is impossible to draw any conclusions from them. Moeckel and Rost (1) concluded from rather scanty data, that the diastatic activity of blood in cases of human diabetes is almost invariably higher than normal. Myers and Killian (2) showed that, in the same disease, the diastatic activity of the blood may be from 2 to 4 times as high as in normal individuals. They also found a close relationship between the blood sugar and the diastatic activity. They concluded that an increase in blood diastase may constitute a very early sign of impending diabetes, and that a fall may afford a more reliable guide to the efficacy of dietetic treatment of this disease than either the blood or urine sugar. De Niord and Schreiner (3) were able to confirm these results, showing in addition that in cases of diabetes complicated by syphilis, the diastatic activity of the blood may be subnormal. On the other hand, Lewis and Mason (4) found that a severe case of diabetes may have a very low blood diastase and showed that there is no relationship between the blood sugar and the blood diastase. Bainbridge and Beddard (5) found that this enzyme was present in the liver and muscles of diabetic patients and of depancreatized cats, but they made no attempt to determine whether the enzyme concentration was greater or less than in normal animals. They also found that in two cases of diabetes, there was no diastase in the blood. Schmerel (6) showed that the urine diastase was abnormally low in diabetes, but was unable

to trace this to a low blood diastase. Von Strasser (7) found normal blood diastase values in sixteen cases of diabetes. In two cases, one complicated by cholecystitis, the other by arteriosclerosis, he obtained high values. Ottenstein (8) in an extensive study of diastase in skin and blood, found low values for the blood diastase in diabetes, while Markowitz and Hough (9) concluded from their investigation on depancreatized dogs that there is no justification for the use of blood diastase estimations in this disease. Popper (10), Janker (11), Stocks (12), and others (13, 14) have shown that blood diastase estimations are of distinct value as a diagnostic sign in pancreatic disease. Pancreatic injury of an obstructive or inflammatory nature causes a marked rise in the diastatic activity of the blood.

In view of the conflicting nature of these observations, it was deemed necessary to furnish additional evidence regarding the blood diastase level in diabetes and to determine if possible the cause of the wide variation observed by other workers. Myers and Killian (2) suggested that the diastase in the blood may be normally regulated by the internal secretion of the pancreas and later, other workers (15, 16) found that insulin lowers the blood diastase in normal rabbits and dogs. *A priori*, then, it seemed probable that the blood diastase in diabetic patients receiving no insulin would be higher than that in patients receiving insulin therapy.

#### EXPERIMENTAL

Of the different methods used to estimate the diastase in blood, those dependent upon the estimation of reducing sugars formed from starch hydrolysis are the most reliable. Since we have shown in a previous communication (17) that the picric acid method of Myers and Killian (2) holds several advantages over other methods for estimating the reducing sugars formed by the action of the blood diastase, we have used it throughout our investigations. The blood diastase determinations were invariably carried out not more than 30 minutes after the blood was drawn. Normal blood samples were obtained from medical students and workers in this laboratory. Diabetic blood samples were drawn from patients in the Diabetic Clinic at Lakeside Hospital.<sup>1</sup>

<sup>1</sup> We are indebted to Dr. I. C. Hanger, Outpatient Department, Lakeside Hospital, who supplied us with blood samples from diabetic patients, and with information as to the clinical history of those cases.

The blood diastase in normal individuals varies between 13 and 19 (Table I). The average value, 16, is in excellent agreement with the results of Myers and Killian (2) who found most of their normal values to lie between 15 and 17.

The blood diastase range in diabetic patients receiving insulin treatment is given in Table II. These patients received the insulin injections about 4 hours before the blood samples were drawn. The blood diastase values vary from 7.2 to 31.8 with an average value of 18.8. Sixteen out of twenty-seven cases are within, or below, the normal range. There would not appear to be any relationship between the dose of insulin injected and the blood diastase level.

TABLE I  
*Blood Diastase\* in Normal Individuals*

Subject	Blood sugar	Blood diastase	Subject	Blood sugar	Blood diastase
	<i>mg. per 100 cc.</i>			<i>mg. per 100 cc.</i>	
S. B. ....	108	13.6	A. Q. ....	110	15.8
M. S. ....	101	14.6	B. B. ....	116	16.1
B. G. ....	113	14.7	R. G. ....	118	17.3
D. C. ....	106	14.7	E. R. ....	114	17.7
A. S. ....	111	14.9	P. F. ....	117	18.1
J. A. S. ....	116	15.2	F. B. ....	106	19.2
Average of 12 subjects.....					16.0

\*As our index or unit in diastase estimations in blood we have employed the per cent of starch (10 mg.) converted to reducing sugar by the amount of blood used (2 cc.)

The results obtained from diabetic cases on dietetic but not on insulin treatment are given in Table III. The blood diastase values vary between 16.1 and 44.3 with an average value of 29.1. Sixteen cases out of twenty are above the normal range. The results also suggest that there may be some relationship between the blood sugar and the blood diastase, a high blood sugar, usually but not invariably, being accompanied by a high blood diastase, and *vice versa*.

Several of the diabetic patients showed symptoms of nephritis, and in view of the findings of Myers and Killian (2) that the blood

diastase is high in this disease, it was considered necessary to indicate those cases in which nephritis might be a complicating

TABLE II  
*Blood Diastase\* in Diabetic Patients Treated with Insulin*

Patient	Insulin	Blood sugar	Blood diastase	Remarks
	<i>units</i>	<i>mg per 100 cc</i>		
L. S	5	278	13 5	Possible nephritis
S S	5	208	19 1	
W. M	10	113	20 6	
A C	10	292	18 6	
H N	10	207	17 8	Severe “
V Z	10	269	13 9	Probable “
R C	10	159	21 3	
E K	15	221	21 2	“ “
W D	15	310	23 4	
F. R	20	331	31 8	
F S	20	283	14 2	“ “
H R	20	294	24 7	
R R	20	217	12 9	
S R	20	413	24 6	Severe “
H. L	20	216	28 6	Mild “
T. B	20	298	18 3	
F. E.	25	232	8 1	Syphilis
A B..	25	217	13 2	Probable nephritis
A. S	30	273	25 8	
R W.	30	416	13 9	Possible “
W. S	30	266	11 4	
A G	35	214	19 4	
T. P	40	298	20 9	
A. S	45	319	7 2	
F. S	45	343	17 4	
M. V	60	116	27 0	
A. C	70	205	18 3	
Average of 27 diabetic patients			18 8	

\*See note to Table I

factor (Tables II and III). Since the occurrence of nephritis is as great (if not greater) in Table II as in Table III, it would not seem that this factor affects our data to any marked extent.

## DISCUSSION

The results of our investigations on diabetic patients, receiving dietetic but not insulin treatment, support the findings of Myers and Killian (2). In these cases, the blood diastase is almost invariably high. There would also appear to be some relationship between the blood sugar and the blood diastase. When insulin

TABLE III  
*Blood Diastase\* in Diabetic Patients Untreated with Insulin*

Patient	Blood sugar <i>mg per 100 cc</i>	Blood diastase	Remarks
R K	141	16 1	Probable nephritis
E K	158	16 3	
A K	163	16 9	
M S	136	19 4	
A K	269	20 1	
M S	224	20 3	Possible nephritis Syphilis
A K	127	21 8	
A. K	252	23 0	
W S	202	28 9	
C K	281	29 1	
M H	188	30 5	Severe nephritis
A Z	190	30 7	
A F	263	32 9	
D M	164	34 1	
M L	203	34 4	
H B	234	38 0	Possible " "
A K	448	40 8	
C B	341	41 7	
M M	280	43 1	
M H	324	44 3	
Average of 20 diabetic patients		29 1	

\*See note to Table I

treatment is resorted to, however, normal or even subnormal blood diastase values are almost always found. We are unable to explain the disagreement between our findings and those of Ottenstein (8). Reid and Narayana (15) and Cohen (16) have shown that in normal animals, insulin causes a decrease in blood diastase and an increase in liver diastase. It is probable, as these workers suggest, that the diastase in the blood fulfils no function in car-

bohydrate metabolism, but that when recalled into the liver cells, it may exert its enzymatic function with regard to the glycogen-glucose transformation. Insulin would appear to play some part in the transfer of the enzyme from the blood to the liver cells.

Diastase in the animal body is generally regarded as being a starch or glycogen-splitting enzyme. It is tentatively suggested, however, that in the presence of insulin, diastase may be reversible in its action and play some part in the formation of glycogen. In the diabetic patient receiving no insulin, there is a high blood sugar, due in part, at least, to breakdown of liver glycogen under the action of diastase. In order to conserve its supply of glycogen, the liver reduces its enzyme content, which is accordingly liberated into the blood. When insulin is administered, the diastase in the blood is recalled into the liver cells, where it may act in a reverse way by converting glucose into glycogen.

#### SUMMARY

The diastatic activity of the blood in normal individuals varies between 13 and 19 (Myers-Killian method).

In diabetic patients, receiving dietetic but not insulin treatment, the diastatic activity of the blood is almost invariably higher than normal. In these cases, also, there appears to be some correlation between the blood sugar and the blood diastase.

In insulin-controlled diabetic patients the diastatic activity of the blood generally approximates the normal.

It is suggested that the diastase while in the blood plays no part in carbohydrate metabolism, and that it is only when, in the presence of insulin, it is recalled into the liver cells that it exerts its enzymatic function with regard to the glucose-glycogen reaction.

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## STUDIES ON ANIMAL DIASTASES

### V. BLOOD AND TISSUE DIASTASES, WITH SPECIAL REFERENCE TO THE DEPANCREATIZED DOG

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(Received for publication, September 19, 1932)

In the preceding paper (1)<sup>\*</sup> we showed that the blood diastase of diabetic patients, untreated with insulin, was generally above normal, while that of patients receiving insulin therapy, on the other hand, was generally normal or subnormal. It was thought that it would be of interest to ascertain the effect of insulin on the blood diastase of depancreatized dogs. The results of previous investigations in this field yield rather conflicting results. Milne and Peters (2) and Myers and Killian (3) found that the blood diastase of dogs increased after pancreatectomy. Karsner, Koechert, and Wahl (4) state that there is an increase after the pancreas is completely removed, but that there is no correlation between the blood diastase and the blood sugar. Reid and Narayana (5) found that pancreatectomy had little or no effect upon the blood diastase level. The slight decrease, which they observe, they attribute to interference of the high blood sugar with the action of the enzyme on the starch substrate. Davis and Ross (6), Otten and Galloway (7), and Markowitz and Hough (8) find a definite decrease in the blood diastase after the operation. The latter workers also report that treatment with insulin brings the blood diastase level back to normal. Since, in human diabetes, insulin would appear to have the opposite effect, it was considered desirable to obtain more data in this regard. In addition, the effect of insulin upon the blood and tissue diastases of normal dogs and rabbits was determined.

#### EXPERIMENTAL

*Experiments on Depancreatized Dogs*—The blood diastase level in six completely depancreatized dogs (depancreatized by J.P.Q.)

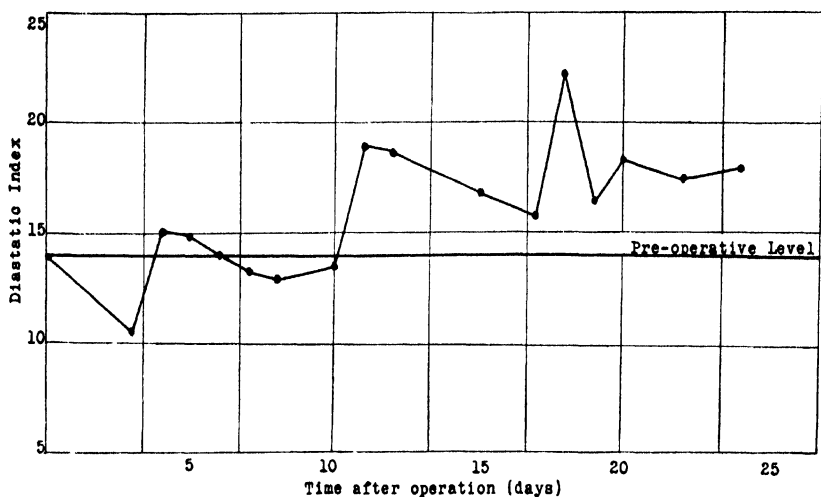


FIG. 1. Blood diastase of dog after pancreatectomy. The animal was kept alive with insulin (18 units daily).

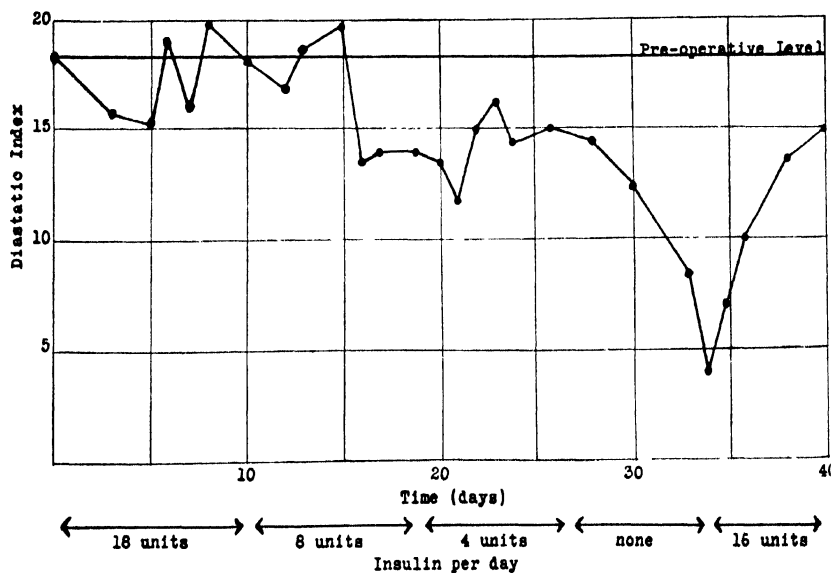


FIG. 2. Effect of insulin on blood diastase of depancreatized dog

was determined at intervals of 2 days over a period of 4 to 6 weeks. Subsequent autopsy in all cases revealed no trace of pancreas tissue. Two of the animals received choline hydrochloride (1 gm. *per os*) daily, in an attempt to prevent excessive fat deposition in the liver (9). In the other cases, raw ox pancreas or pancreatin was given with the food, which consisted of bread and meat scraps. It would not appear from our results that the feeding of either choline or pancreas has any effect on the blood diastase.

TABLE I

*Effect of Insulin on Blood, Liver, and Muscle Diastase of Normal Dog*

Dog No.	Weight	Remarks	Blood sugar	Diastase*		
				Blood per 0.2 cc.	Liver per gm.	Muscle per gm.
	<i>kg.</i>		<i>mg. per 100 cc.</i>			
1. Control experiment, amytal anesthesia, no insulin	17.8	Before anesthesia	95	17.8		
		After " "	113	18.6	58.4	18.6
		2 hrs. after anesthesia	115	16.9	62.4	19.8
2. Amytal anesthesia, 2 units insulin per kilo subcutaneously	19.5	Before insulin	95	14.8	51.6	
		2 hrs. after insulin	77	9.6	98.3	
3. Amytal anesthesia, 2 units insulin per kilo subcutaneously	15.5	Before anesthesia	86	19.9		
		After " "	106	19.3	36.8	14.1
		4 hrs. after insulin	76	15.9	60.5	15.7

\*As our index or unit in diastase estimations in blood we have employed the per cent of starch (10 mg.) converted to reducing sugar by the amount of blood used (0.2 cc.).

For the blood diastase estimations, which were carried out within half an hour of the withdrawal of blood, the method of Myers and Killian (3) was employed, and for the blood sugar, Folin's (10) micro method. Since in all six cases, essentially similar results were obtained, the data from one typical experiment only will be reported. Fig. 1 represents the effect of pancreatectomy upon the blood diastase of a dog in which the diabetes was controlled by daily insulin injections. Apparently the blood diastase de-

creases during the first few days after the operation, but subsequently, it rises to the preoperative level or even higher. At no time does the blood diastase completely disappear. It would appear, therefore, that the pancreas cannot be the only source of diastase in the blood as has been suggested (11, 12).

When the dosage of insulin is progressively reduced, the diastatic activity of the blood decreases, the effect being accentuated on complete cessation of insulin treatment (Fig. 2). The blood diastase again increases when insulin administration is resumed.

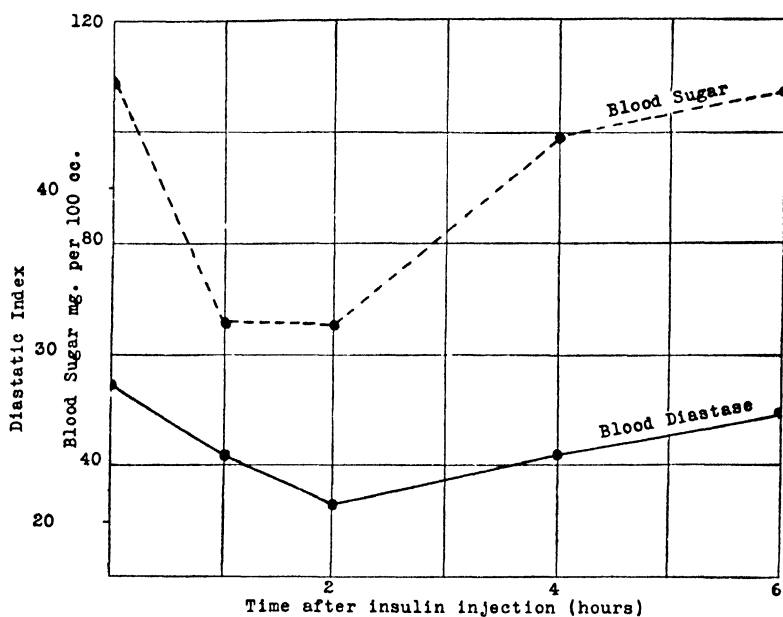


FIG. 3. Effect of insulin on blood diastase of rabbits (no hypoglycemic convulsions).

*Experiments on Normal Dogs*—The effect of insulin on the blood and tissue diastases of normal dog was investigated. In these experiments, after complete amytal anesthesia, samples of blood, liver, and muscle were taken before and after subcutaneous injection of insulin. The results are given in Table I. The first experiment (Dog 1) was a control, devised in order to determine the effect (if any) of amytal anesthesia upon the blood and tissue

diastases. Our data indicate that amytal has no effect on the diastatic activity of either blood or tissue. In Dogs 2 and 3, which received insulin after amytal anesthesia, there is a fall in

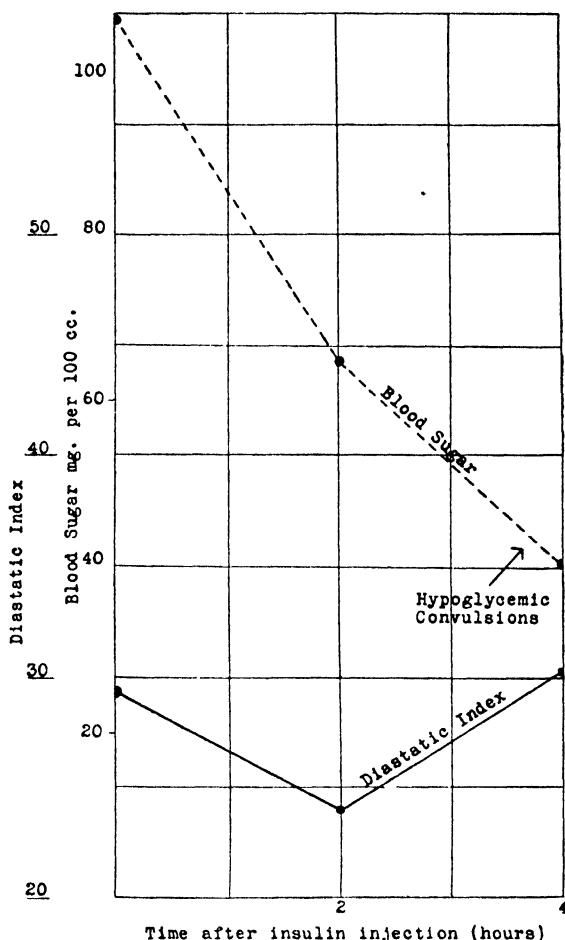


FIG. 4. Effect of insulin on blood diastase of rabbit (hypoglycemic convulsions).

the blood diastase, a marked rise in the liver diastase, and no change in the muscle diastase.

*Experiments on Normal Rabbits*—When half the dosage of

insulin necessary to produce hypoglycemic convulsions is administered daily to normal rabbits over a period of 10 days, and the diastatic activity of the blood determined before each insulin injection, no change in the enzyme concentration is observed. If the blood diastase is determined a few hours after an injection of insulin, however, a slight but appreciable decrease takes place (Fig. 3). The blood diastase curve follows a course approximately parallel to that of the blood sugar. It is apparent, then, that the diastatic activity of the blood returns to the normal level when the effect of the insulin has worn off.

When the insulin dosage is increased in order to produce hypoglycemic convulsions, the blood diastase at first decreases, then, when convulsions occur, rises sharply to the preexperimental level (Fig. 4). These results are in precise agreement with those of other investigators (5).

#### DISCUSSION

Our results show conclusively that pancreatectomy in dogs, unaccompanied by insulin administration, causes a lowering in the blood diastase. If the animals are kept alive with insulin, the blood diastase, after a drop during the first few days after the operation, rises to and may exceed the preoperative level. Withdrawal of insulin treatment invariably causes the diastatic activity of the blood to decrease markedly. On resuming insulin therapy, we find that the blood diastase returns to the normal level. Our data, therefore, confirm those of Markowitz and Hough (8). The latter workers, however, conclude that insulin has a very variable effect on the blood diastase, whereas our results leave us in no doubt but that its effect, in depancreatized dogs, is to raise the diastatic activity of the blood. We have, therefore, failed to substantiate our findings in the case of human diabetes (1). Why this should be the case is not yet clear.

It may be noted, however, that there are two important differences between the blood diastase findings in the human diabetic and the depancreatized dog. Although the blood of the depancreatized dog may exhibit practically normal diastatic activity when the carbohydrate metabolism is regulated with insulin, thus showing that the pancreas is not normally the only source of the blood diastase, it must still be borne in mind that the

depancreatized dog is completely deprived of his external pancreatic secretion, while this is not necessarily impaired in the diabetic human. That the external secretion of the pancreas may influence the blood diastase is evident from the fact that the diastatic activity of the blood may be considerably increased in pancreatic disease, thus suggesting an absorption of pancreatic amylase into the blood stream. Johnson and Wies (13) have recently shown that ligation of the pancreatic ducts in the dog produces a marked increase in serum amylase. This may be the explanation of the difference in the blood diastase findings in the human diabetic and the depancreatized dog, although insufficient data are available to prove this hypothesis. It should further be borne in mind that the diastatic activity of dog blood is about 10 times that of human blood.

It is apparent that as long as insulin is administered to depancreatized dogs, the blood diastase remains normal or even higher than normal. Even when insulin therapy is withdrawn, the diastase never disappears entirely from the blood. We conclude, therefore, that there must be a source or sources of blood diastase, apart from the pancreas. What these sources are, has not been determined in this investigation.

It has been stated in the literature (14) that choline hydrochloride has a stimulating action on pancreatic amylase. From our data on depancreatized dogs, it would not appear that the oral administration of either choline hydrochloride, fresh raw pancreas, or pancreatin had any appreciable effect on blood diastase. Incidentally, it may be mentioned that those animals receiving choline hydrochloride mixed with their food showed on autopsy much less deposition of fat in the liver, thus supporting the observations of Best, Hershey, and Huntsman (9).

Our experiments on normal dogs show that, after insulin treatment, the diastase decreases in the blood, increases in the liver, and remains unchanged in the muscles. This is in agreement with the data of Cohen (15). McGuigan (16) believes that the diastase content of tissue may be a measure of its functional activity and of basal metabolism. Our results support his hypothesis in some measure. Reid and Narayana (5) showed that, in dogs, insulin injections brought about a decided fall in the blood diastase and that, coincident with the fall of the blood sugar to the

hypoglycemic level, there was a sharp rise in the blood diastase to the preexperimental figure or above it. Using normal rabbits, we have confirmed their results. It would appear that, in normal animals, administration of insulin causes a reabsorption of the diastase in the blood into the liver cells. It is tentatively suggested that the presence or absence of insulin may, in part at least, determine the direction of the glycogen-glucose reaction. When the insulin content of the blood and tissue is low, as in human diabetes, liver glycogenolysis takes place. To suppress this reaction as much as possible, the liver liberates its diastase into the blood where the enzyme is inactive. After an insulin injection, however, the diastase in the blood passes into the liver cells. If we suppose that, in the presence of insulin, the enzyme assists in the formation of glycogen from glucose, liver glycogen will increase, which is actually what occurs. Our investigations on depancreatized dogs, however, do not bear out this hypothesis.

#### SUMMARY

In depancreatized dogs, the blood diastase decreases during the first few days after the operation but returns to the preoperative level or above it if the diabetic condition is controlled by insulin.

Cessation of insulin treatment causes a decrease in the blood diastase, with a subsequent return to normal when the insulin injections are resumed.

In normal dogs, insulin causes a fall in the blood diastase, an increase in the liver diastase, and no change in the muscle diastase.

In normal rabbits, insulin causes the blood diastase to decrease. When hypoglycemic convulsions occur, the blood diastase increases sharply to the preexperimental level.

It is suggested that in the normal animal insulin causes a recall of the diastase in the blood into the liver cells, where, and where only, the enzyme fulfils its rôle in carbohydrate metabolism.

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## AN ALTERNATIVE REAGENT FOR THE ESTIMATION OF GALACTOSE

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In a previous paper Harding and Grant (1) gave the details of a method for the determination of galactose, applicable to human bloods and urines. They showed that bakers' yeast, adapted or acclimatized to the fermentation of galactose, could be used for its quantitative determination. As a result of some private criticism, and observations, it became evident that "galac" yeast might remove small amounts of maltose. The amounts were not large, never more than 2 to 3 mg. out of a 20 mg. per cent solution, sometimes none at all; nevertheless, such occasional findings cast a slight uncertainty on the specificity of our reagent. Nor could we be certain of the action of ordinary bakers' yeast upon maltose. Raymond and Blanco (2) had reported no removal action on that sugar under their conditions, a finding which we had confirmed; but later experiments showed that, under other conditions, considerable but variable amounts might disappear.<sup>1</sup> We were thus faced with the situation that we could neither depend on ordinary bakers' yeast to remove maltose completely, nor on galac yeast invariably to remain inert.

Such criticisms do not affect the use of galac yeast for the original purpose for which it was intended; *viz.*, the estimation of galactose in blood, urine, and tissues in an effort to follow the course of metabolism of that sugar after its oral administration. Maltose, while possibly a transitory intermediate in some phases of carbohydrate metabolism, does not accumulate in the mammalian body to such an extent that it can be recognized by any

<sup>1</sup> The details of some of these experiments and a method for the estimation of maltose will be published shortly.

present day methods. A few cases of maltosuria have been reported but these occur only under exceptional conditions. The results of Harding and Grant on normal bloods and urines were negative for galactose. They are thus unaffected by these criticisms.

*Saccharomyces marxianus* is a reagent for galactose devoid of action on maltose. When cultured and used under our experimental conditions, it removes glucose, fructose, mannose, sucrose, and galactose. It has no action on maltose, lactose, arabinose, or xylose. Save for the difference towards maltose, its behavior to sugars is the same as galac yeast. It can be used under the same conditions. 0.5 gm. of washed *Saccharomyces marxianus* removes completely the galactose from 10 cc. of a 20 mg. per cent solution. It is applicable to Folin-Wu blood filtrates, and to urines diluted and cleared with  $H_2SO_4$  and Lloyd's reagent. If glucose, fructose, and mannose are first removed from solution, any further loss in reducing power following the use of *Saccharomyces marxianus* becomes specific for galactose. We have not investigated its keeping properties over any length of time, having always used it within 24 hours of its preparation.

Under conditions where the presence of maltose may be suspected, *Saccharomyces marxianus* is therefore a reagent for galactose superior to galac yeast. Under more limited conditions galac yeast is perhaps to be preferred. Its good keeping properties (provided it is washed daily) make it useful when a large number of daily analyses have to be carried out, as in a series of galactose tolerance tests. During the last year Standard Brands Incorporated<sup>2</sup> have prepared for us quantities of galac yeast. Their preparations have been much superior to those made by Harding and Grant, in their removal power towards galactose. All of them, however, possessed a slight removal action on maltose.

#### EXPERIMENTAL

*Strain*—*Saccharomyces marxianus*, No. 2343 American Type Culture Collection<sup>3</sup> was used.

<sup>2</sup> We wish to acknowledge the assistance we have received from Dr. R. E. Lee of the Research Division and Mr. Charles N. Frey of the Manufacturing Division of Standard Brands Incorporated, New York.

<sup>3</sup> Obtainable from John McCormick Institute for Infectious Diseases, Chicago.

*Cultural Conditions*—Inoculate into glucose peptone broth. Let growth proceed for 2 days at 38°. Plate on glucose agar or galactose agar and let growth continue for 6 days at 38°. There

TABLE I  
*Comparison of Removal Action of Galac Yeast and Saccharomyces marzianus on 20 Mg. Per Cent Solution of Various Sugars*

Sugar	After galac yeast	After <i>Saccharomyces marzianus</i>
	mg. per 100 cc.	mg. per 100 cc.
Glucose.....	0.0	0.0
Fructose.....	0.0	0.0
Mannose.....	0.0	0.0
Galactose.....	0.0	0.0
Maltose.....	18.2	20.0
Lactose.....	20.0	19.2
Saccharose.....	0.0	0.0
Arabinose.....	19.8	19.3
Xylose.....	19.2	19.8

TABLE II  
*Removal Action of Saccharomyces marzianus on Various Sugars Added to Fermented Folin-Wu Blood Filtrates and Normal Human Urines*

Sugar	After <i>Saccharomyces marzianus</i>	
	Urine*	Blood†
	mg. per 100 cc.	mg. per 100 cc.
Glucose.....	0.04	0.0
Fructose.....	0.08	0.1
Mannose.....	0.50	0.2
Galactose.....	0.20	0.0
Maltose.....	19.70	10.3
Lactose.....	19.60	10.2

\* 20 mg. per 100 cc. were added to urine. Figures are the average of five determinations.

† 10 mg. per 100 cc. were added to fermented Folin-Wu blood filtrate.

is a vigorous growth in the first 48 hours, but preparations made at this period show poor galactose-removing properties. We have found flat 26 ounce narrow necked bottles very convenient for growth.

*Preparation*—Wash the 6 day growth off the agar with a stream of distilled water. Centrifuge the yeast at about 1500 R.P.M. Wash and recentrifuge until washings are clear and free from reducing material. Make a 50 per cent wet weight suspension with water. Use within 24 hours. Yield, about 2 gm. of washed wet weight yeast per 26 ounce bottle.

*Use*—1.0 cc. of the 50 per cent suspension is placed in a clean dry 15 cc. centrifuge tube, centrifuged, the water poured off, and adherent moisture removed by filter paper. 10 cc. of the sugar solution to be tested are added to the yeast, well mixed, and incubated 30 minutes at 38° with continued stirring. Cool, and remove the yeast by centrifuging. The procedures for the determination of total sugar and residual sugar (including controls) are the same as in the use of galac yeast.

A comparison of the removal action on different sugars of galac yeast and *Saccharomyces marxianus* is shown in Table I. The recovery of added sugars from Folin-Wu blood filtrates and urines is shown in Table II.

Urines were diluted and treated with H<sub>2</sub>SO<sub>4</sub> and Lloyd's reagent before estimation. All urines were from fasting subjects and were free from fermentable sugar.

#### SUMMARY

*Saccharomyces marxianus* can be used to determine galactose in mixtures of certain sugars in aqueous solutions, in urines, and in Folin-Wu blood filtrates.

It is devoid of action on maltose, in contrast to galac yeast which sometimes shows a small removal.

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## METABOLISM OF GALACTOSE

### I. CUTANEOUS BLOOD SUGARS AFTER GALACTOSE INGESTION

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Our knowledge of the fate of galactose after its oral ingestion has been limited by the analytical methods at our disposal. As a reducing sugar, the galactose could be traced by earlier workers only by methods common to those for glucose. The use of bakers' yeast enabled these two sugars to be separated. By this means, Blanco (1), Corley (2), and Harding and van Nostrand (3) reduced the number of assumptions used in the interpretation of galactose metabolism. Roe and Schwartzman (4) by using Benedict's (5) reagent for blood sugar after fermentation have minimized the occurrence of non-sugar reducing substances in the fraction analyzed as galactose.

In two papers from this laboratory methods have been developed for the determination of galactose in bloods and urines. Both "galac" yeast (6) and *Saccharomyces marxianus* (7) quantitatively remove galactose from Folin-Wu blood filtrates and from urines previously treated with  $\text{H}_2\text{SO}_4$  and Lloyd's reagent. The total reducing power of these liquids can be fractionated into (a) fermentable sugars, (b) galactose, and (c) residual reducing substances. It is thus possible to follow the course of galactose in blood and urine after its oral ingestion in man, and to trace its influence, if any, on the other fractions. We have been able to gather further information on its behavior under varying conditions and to clarify many minor points of dispute. Our progress has been helped by our ability to apply this method of sugar fractionation to small amounts of cutaneous blood. All previous work either has been with venous blood, if the separation of fermentable sugar from the remaining reducing substances has

been attempted by bakers' yeast, or has depended on the determination of total reducing substances in cutaneous blood.

### *Analytical Methods*

#### *Fractionation of Reducing Substances in Cutaneous Blood*

*Folin-Wu Preparation of Cutaneous Blood*—0.5 cc. of cutaneous blood<sup>1</sup> is drawn into a capillary pipette, washed into a centrifuge tube with 7.5 cc. of 0.66 per cent sodium tungstate solution, and 2.0 cc. of 0.16 N H<sub>2</sub>SO<sub>4</sub> are added. The mixture is well stirred with a thin glass rod, allowed to stand 10 to 15 minutes, and centrifuged.

*Total Reducing Substances*—Two 1.0 cc. portions of the Folin-Wu centrifugate are removed and placed in 6 ×  $\frac{3}{4}$  inch test-tubes. To each is added 1.0 cc. of the Harding and Downs (8) modification of the Shaffer-Hartmann sugar reagent. The tubes are stoppered with absorbent cotton, heated exactly 10 minutes in a rapidly boiling water bath, cooled to 30°, 1.0 cc. of 1 per cent KI solution is added, and 1.0 cc. of 1 N H<sub>2</sub>SO<sub>4</sub>. The excess I<sub>2</sub> is titrated with 0.0025 N thiosulfate, with the use of starch in saturated phenol red as indicator. A control experiment with water instead of Folin-Wu centrifugate is carried out alongside the determination. This method is accurate to  $\pm 0.02$  cc. of thiosulfate, corresponding to less than 4 mg. of glucose per 100 cc. of blood.

The Harding and Downs modification of the Shaffer-Hartmann reagent is very similar to that described by Spannuth and Power (9). It possesses the advantage over previously described modifications in giving lower figures for the non-fermentable reducing fractions of blood and urine, and in only requiring one control tube for each batch of sugar determinations.

*Fermentable Sugar*—0.25 gm. (wet weight) of washed bakers' yeast is added to the remainder of the Folin-Wu centrifugate and incubated 10 minutes at 37° with constant stirring. The yeast is centrifuged and the supernatant liquid poured into a clean dry centrifuge tube. Any stray cells in this liquid are removed by a second centrifugation. Two 1.0 cc. portions are then removed

<sup>1</sup> Suitable pipettes have been made for us by Messrs. Eimer and Amend, New York.

for analysis, by the method described in the previous paragraph. The difference represents fermentable sugar.

*Galactose*—0.5 gm. (wet weight) of washed galac yeast or *Saccharomyces marxianus* is added to the remaining portion of the Folin-Wu centrifugate and incubated 20 minutes at 37° with constant stirring. The yeast is removed by centrifugation and two 1.0 cc. portions are removed for analysis as just described.

TABLE I  
*Galactose Tolerance Tests (Series A)*

Subject	Blood glucose, mg. per 100 cc.							Blood galactose, mg. per 100 cc.							Urine galactose
	Min.							Min.							
	0	15	30	45	60	90	120	15	30	45	60	90	120		
														mg.	
R. F.....	90	89	98	96	92		83	4	0	7	8		0	56	
H. P.....	98		95		92	84	92		5		9	0	0	89	
G. B.....	94		93		88	90	94		0		11	0	0	147	
S. C.....	85	97	87	84	82		75	10	9	11	0		0	158	
W. S.....	81	91	92	90	85		79	4	12	13	10		0	211	
W. F.....	86		96		90	85	76		11		13	0	8	232	
R. G.....	90		91		89	85	87		19		19	12	0	249	
J. S.....	99	126	125	114	101		85	0	9	0	0		0	260	
T. T.....	83	103	108	114	81		72	0	21	18	18		0	326	
G. H.....	84	90	89	84	87		74	20	32	28	21		0	436	
M. M.....	80	87	82	80	79		74	16	23	22	8		0	471	
G. A. G.....	91		100		93	90	92		22		31	0	0	627	
G. R.....	90	96	88	99	83		86	0	21	24	12		0	693	
C. E. D.....	89	111	83	83	88		91	20	62	65	42		8	1090	
A. R. A.....	80	108	91	83	82		77	16	45	54	40		6	1248	
R. J.....	83		92		79		70		51		70		0	1766	

This gives the *residual reducing substances*. The difference gives galactose.

#### *Fractionation of Reducing Substances in Venous Blood and Urine*

Venous blood was fractionated in accordance with the methods just given for cutaneous blood. Urines were fractionated by the methods of Harding and Grant (6). All results reported in this paper are given as glucose. All difference values of 0.03 cc. of thiosulfate or less are reported as zero.

## EXPERIMENTAL

Our observations divide themselves into five series.

*Series A. Fractionation of Cutaneous Blood and Urine Sugars after Ingestion of 40 Gm. of Galactose by Fasting Subjects*—The galactose was given at 9.00 a.m. in 400 cc. of water. Sixteen normal male subjects were used in this series. The subjects took no food after 6.00 p.m. the previous evening. The night urine was discarded at 7.00 a.m. and the 7.00 to 9.00 a.m. urine analyzed as a control. Blood specimens were taken every 15 minutes

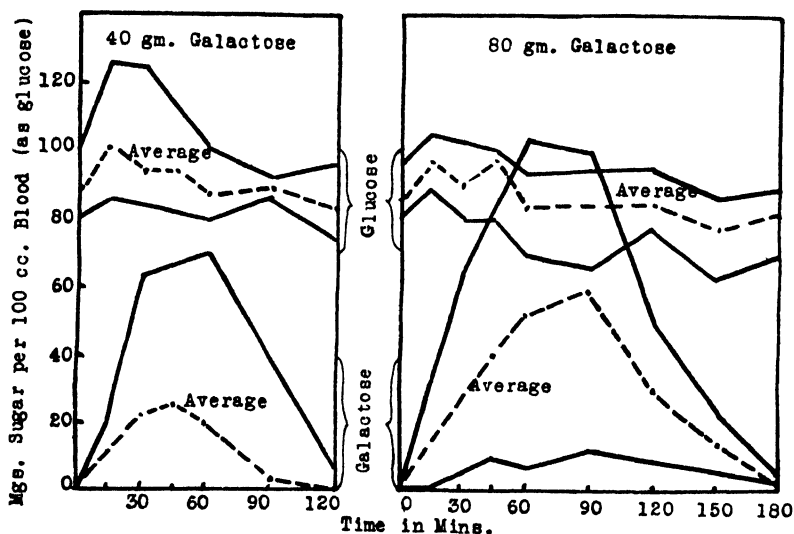


FIG. 1. Maximal, average, and minimal blood sugars after the ingestion of 40 gm. of galactose and 80 gm. of galactose. Average urine galactose after 40 gm. = 500 mg. Average urine galactose after 80 gm. = 2650 mg.

in the 1st hour and at 30 minute intervals in the 2nd hour. A urine collection was again made at 11.00 a.m. Detailed results are given in Table I, composite results in Fig. 1.

*Series B. A Series of Determinations Similar to Series A, 80 Gm. of Galactose Being Administered*—The blood and urine collections were continued till 12.00 noon. Composite results on six subjects are shown in Fig. 1.

*Series C. Arterial-Venous Differences in Blood Glucose and*

*Galactose after Ingestion of 40 Gm. of Galactose under Conditions Similar to Series A—Results on four subjects are shown in Fig. 2.*

*Series D. The Effect of Added Glucose (30 Gm.) on the Blood and Urine Sugars after Ingestion of 40 Gm. of Galactose under the Fasting Conditions of Series A—Results on six subjects are shown in Fig. 3.*

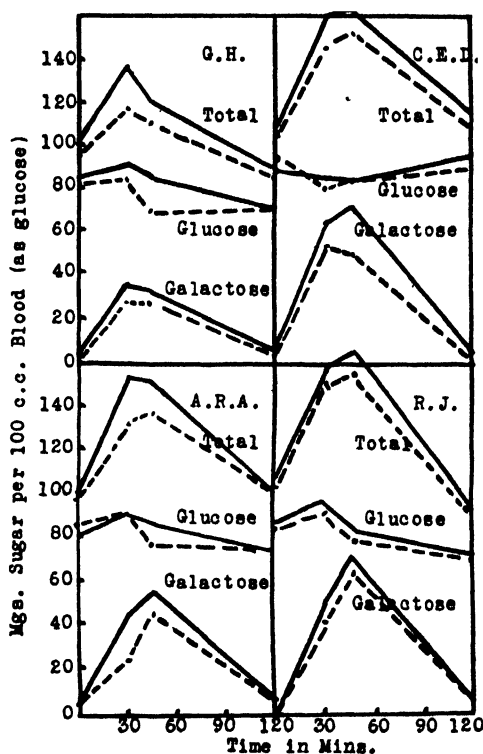


FIG. 2. Arterial-venous differences in blood sugars after the ingestion of 40 gm. of galactose. The solid line represents arterial blood; the broken line, venous blood.

*Series E. Fractionation of Cutaneous Blood and Urine Sugars after Ingestion of 40 Gm. of Galactose at 4.00 P.M.—The subjects took lunch at 12.00 noon. The urine passed from 2.00 to 4.00 p.m. was analyzed in addition to urine voided from 4.00 to 6.00 p.m. The noon meal was varied, being mixed, high carbohydrate,*

or high fat. The details of the carbohydrate and fat meal are given by Harding and Selby (10). Results on four subjects are shown in Figs. 4 and 5. The curves during fasting for the same individuals occur in Table I.

We have given a fixed dose of galactose, rather than one fluctuating with body weight, or body surface, though the latter may

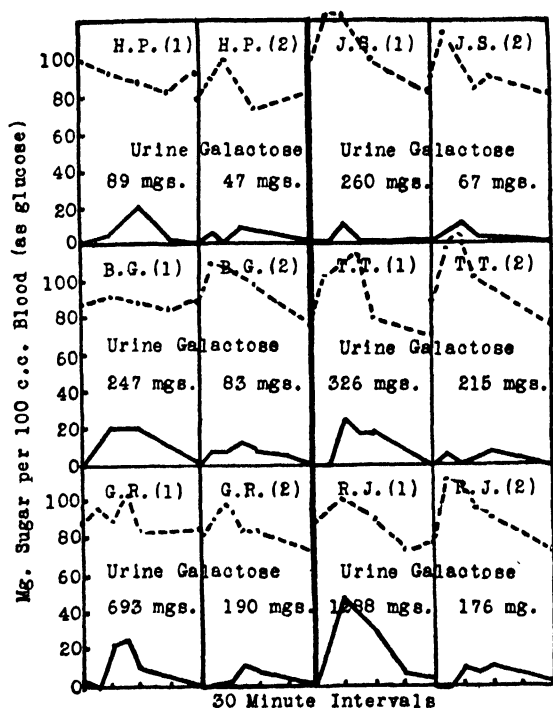


FIG. 3. Showing the effect of ingestion of 30 gm. of glucose with 40 gm. of galactose on blood and urine glucose and galactose. (1) indicates 40 gm. of galactose; (2), 40 gm. of galactose + 30 gm. of glucose. The solid line represents galactose; the broken line, glucose.

seem more logical and scientific. From time to time arguments have been advanced in favor of varying the intake of glucose in the glucose tolerance tests. The results obtained with such regulated amounts of glucose have, however, proved disappointing from the standpoint of uniformity. The blood sugar

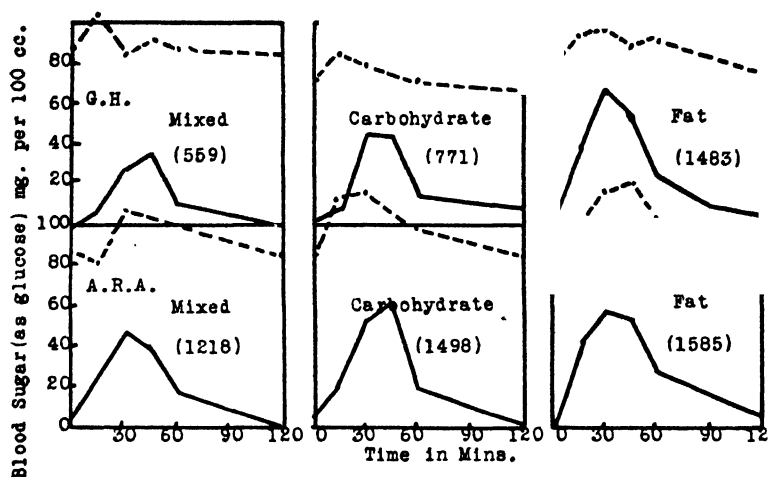


FIG. 4. Galactose tolerance tests at 4.00 p.m. The nature of the noon meal is shown for each subject. The figures in parentheses represent the urinary galactose in mg. for each experiment. The solid line represents galactose; the broken line, glucose.

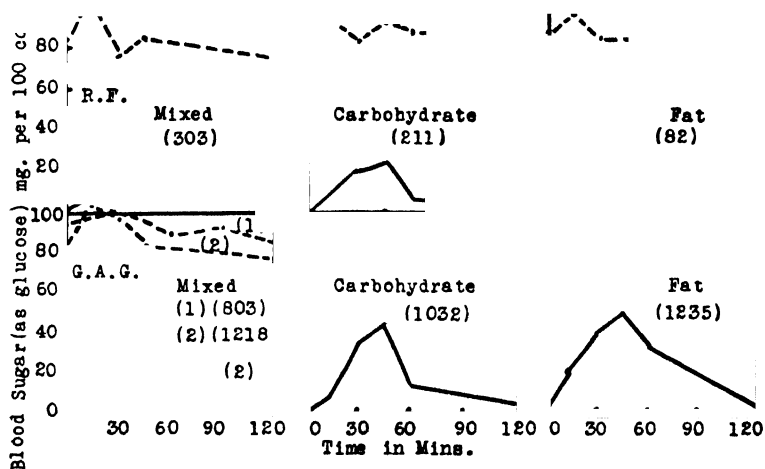


FIG. 5. Galactose tolerance tests at 4.00 p.m. The nature of the noon meal is shown for each subject. The figures in parentheses represent the urinary galactose in mg. for each experiment. The solid line represents galactose; the broken line, glucose. Two observations were made on subject G. A. G.

curves show just as great a variation as after a fixed intake of 50 or 100 gm. The same would appear to be true with galactose. If the results of Harding and van Nostrand, who used a fixed intake of 50 gm. of galactose, are compared with those of Roe and Schwartzman, who used a regulated dose of 1 gm. per kilo of body weight, it will be seen that both show large fluctuations. Our choice of 40 gm. as the fixed intake of galactose rather than 50 gm. has been influenced by the recent German and English work on galactose tolerance tests in hepatic disorders.

#### DISCUSSION

*Blood Galactose*—In a small series of experiments Harding and Grant have shown the absence of galactose in normal blood from fasting subjects. These results are confirmed in the present work. After galactose ingestion, however, galactose was observed in all bloods. The amount in some cases was very small and its presence transient. It was always present in the 30 or 60 minute sample. This result is supplementary to that of Harding and van Nostrand whose methods did not enable them to detect very small amounts of galactose in venous bloods, although there was a marked increase in the non-fermentable reducing fraction of the urine. Only hourly specimens were taken by Harding and van Nostrand. This and the possibility of arterial-venous differences may explain the apparent lack of agreement.

Galactose tolerance tests for disorders of hepatic function were first suggested in 1906 by Bauer (11). The criterion was the amount of galactosuria. Such tests received little or no general acceptance in England or America; indeed, so little was their supposed clinical value that in 1925, Greene, Snell, and Walters (12), reviewing the then existent hepatic function tests, dismissed the use of galactose in half a line. Rowe (13) and Rowe and Chandler (14) however believed galactose tolerance tests helpful in certain endocrine disturbances. In 1922, Kahler and Machold (15) determined cutaneous blood sugar at hourly intervals after the ingestion of 40 gm. of galactose. They set a definite limit of a rise of 30 mg. of sugar per 100 cc. of blood as normal. Their standard normal rise appears to have been accepted by Davies (16) and Elmer and Scheps (17), although Bauer and Wozasek (18) had previously pointed out in 1923 that the

total blood sugar might increase by 60 mg. per 100 cc. in occasional cases. Such might still be considered normal and were certainly unconnected with hepatic disorders.

The observations of Series A show that the average blood galactose found after a 40 gm. ingestion reaches 30 mg. In two of these however it is over 60 mg. The use of figures for total sugar instead of galactose can occasionally be misleading. Subjects J. S. and T. T. show marked elevations of glucose. These added to the galactose would give increases of total sugar of 37 mg. instead of 8 mg. of galactose, and 52 mg. of total sugar instead of 18 mg. of galactose respectively. The range of normal variation of either total blood sugar or galactose is evidently wide, and due consideration must be given to such a range in the clinical evaluation of galactose tolerance tests.

The position of the peak may perhaps be of value. It occurs during the 1st hour. This applies to either galactose or total sugar. At the end of 90 minutes the direction of the curve is downward, and has reached the normal fasting zero (or very nearly) in 120 minutes. Kahler and Machold do not give figures for the varying time intervals, but all their normals and those of Bauer and Wozasek show marked diminution from the peak blood sugar at the end of 2 hours. After 50 gm. of galactose were given, thirteen out of fourteen subjects of Harding and van Nostrand showed a blood galactose peak on the 1st hour, if any increase at all was noted. The one exception showed a peak on the 2nd hour. The total venous blood sugar of Foster's (19) subject was also falling between 45 and 75 minutes after an intake of 40 gm. of galactose. It would thus seem a reliable conclusion that, in either arterial or venous blood, the total sugar and the true galactose have reached peak value during the 1st hour and fall to fasting level at the end of 2 hours, if the dose of galactose is moderate in amount. In ordinary clinical galactose tolerance tests it would seem amply sufficient to take 30 minute blood samples.

After the administration of 80 gm. of galactose, both galactemia and galactosuria increase (Fig. 1) (Foster (20); Rowe and Chandler (14)); Reinhold and Karr (21) and Corley (22) believed this to be so, though all but the latter were compelled to rely on the increase in total sugar as a basis for their conclusion. The high

figures of Roe and Schwartzman lead to the same inference. In a discussion of galactemia after galactose ingestion, comparison inevitably is made with the hyperglycemia following glucose ingestion, and many text-books, etc. (21, 23) draw attention to the high blood sugars following the former. Such a comparison is misleading if the amount of ingested sugar is neglected. After 40 to 50 gm. of galactose are ingested, the average blood sugar rise is less than after 40 to 50 gm. of glucose; after 80 to 100 gm. of galactose, it is higher than after the same amount of glucose.

Our interest in possible parallels or discrepancies between glucose and galactose tolerance tests led us to undertake the experiments of Series E. Harding and Selby (10) have shown the occurrence of a mild glycosuria after 50 gm. of glucose are ingested at 4.00 p.m., whereas the same amount of glucose in the morning fasting condition gives no glycosuria. In the majority of subjects the peak of the afternoon blood sugar curve was higher than in the morning. The afternoon glycosuria and hyperglycemia were greatest after a noon meal of fat, but were also observed after high carbohydrate, protein, or mixed meals. Could a similar phenomenon be observed after the administration of 40 gm. of galactose at 4.00 p.m.?

The results (Figs. 4 and 5) fail to duplicate the clear evidence given by glucose. Only one out of four subjects shows marked increases in the afternoon galactosuria and galactemia. The remaining three show no evidence at all of a decreased galactose tolerance at 4.00 p.m. even though the high fat noon meal produced ketosis. Evidently there seems to be no parallel under these conditions between glucose and galactose tolerance tests. Our results after the high fat noon meal should be comparable with results obtained after starvation. Goldblatt (24) found the total blood sugar to rise 55 mg. following the ingestion of 50 gm. of galactose after 42 hours starvation. This falls within the possible normal limits. Reinhold and Karr (21) show for the rabbit blood sugar curves higher after 4 days fasting than after 18 hours but Corley (22) found little effect.

Wierzuchowski *et al.* (25) have investigated the galactose metabolism of dogs under conditions of maximal utilization. The assimilation of galactose was not affected by the type of feeding, by hunger, or by adrenalin or thyroxine. An average of 25 per

cent was metabolized and 74 per cent excreted in the urine under these conditions. Insulin slightly diminished the urine output of galactose. Evidently factors powerful in affecting the removal of glucose play little part in the immediate changes of galactose.

An interesting fact discovered by Folin and Berglund (26) was that for the same dose of galactose, added glucose produced a decreased galactosuria. There has been considerable discussion on the mechanism of this phenomenon. Bodansky (27) claimed decreased hyperglycemia in the dog as a result of the added glucose, whereas Reinhold and Karr (21) show for the rabbit an average blood sugar curve for a mixture of glucose and galactose differing little from galactose alone. Corley (28) found that in the rabbit an intravenous administration of the two sugars did little but increase the excretion of the galactose. He, however, confirmed the decreased galactosuria and galactemia if the mixed sugars were given orally. The most definite evidence is that of Cori and Cori (29), who found a lessened absorption of both glucose and galactose from the gut of the rat, if a mixture was supplied. Our own experiments on men as shown in Fig. 3 can be construed according to the interpretation of Cori and Cori. In all six subjects the amount of galactosuria is decreased if 30 gm. of glucose are added to the standard galactose tolerance test. It happens also that the peak of the galactemia is lower after the mixed sugars are given, in all subjects. In two, however, the heights of the blood galactose curves, both after galactose alone and after galactose + glucose are ingested, are so small, that the decrease in the latter experiments would have no significance, were these the sole results. In the other four subjects the lessened galactemia is very evident. With a lessened galactemia is associated a lessened galactosuria. A lessened galactemia is also associated with a lessened intake. Also, it is to be noted that the increases in blood glucose are very small. From our own experience it would seem an odd series of observations in which, out of six normal subjects, not one showed a maximal blood sugar increase of 35 mg. per 100 cc. after taking 30 mg. of glucose. Yet these are the results produced by a mixture of 30 gm. of glucose + 40 gm. of galactose. Subjects T. T. and G. R. show no more increase in blood glucose after receiving the mixed sugars than after the administration of pure galactose. It seems reasonable

to conclude that comparatively little glucose reached the blood stream.

It has been concluded that galactose is a well utilized sugar when given in moderate amounts (3, 4). In the rat, however, its rate of forming glycogen is much slower than that of either glucose or fructose (30). On the other hand the respiratory quotient approaches unity after its ingestion (31), an increase which does not appear to be due to the liberation of CO<sub>2</sub> due to lactic acid formation, according to Campbell, Maltby, and Soskin (32),

TABLE II

*Residual Reducing Substances in Blood after Removal of Fermentable Sugar and Galactose*

Subject	Glucose, mg per 100 cc.						
	0 min	15 min	30 min	45 min	60 min	120 min	
R. F.	12	11	14	13	13	10	After 40 gm galactose
G. H.	12	14	14	10	10	14	
M. M.	14	13	12	12	12	14	
W. S.	15	17	15	15	17	15	
A. R. A.	15	15	17	15	14	14	
“	12	15	14	10	14	13	After 80 gm galactose
G. H.	18	14	18	19	19	19	
J. S.	15	14	13	13	15	16	
R. F.	14	17	15	15	15	14	
H. P.	15	16	15	13	14	14	

The figures also illustrate the level of the non-sugar reducing substances in whole blood as determined by the new copper reagent.

though Wierzuchowski *et al.* (25) report an increase in blood lactic acid under conditions of maximal utilization. Further light on galactose utilization might be obtained by a study of arterial-venous differences. Cutaneous blood is now commonly accepted as representing arterial blood in composition (33). Foster (19) records two experiments showing a marked absorption by the tissues after galactose ingestion but his figures are those of total sugar, and the difference may well be due to blood glucose. Our results on four subjects are shown in Fig. 2. In all subjects there is a well defined arterial-venous difference in total sugar. Part is due to glucose; part is due to galactose. In two of the subjects

the greater part of the arterial-venous difference is ascribable to galactose. The fact, however, that galactose absorption by the tissues is accompanied by glucose absorption must render questionable attempts to interpret data on the sole basis of direct galactose utilization where the possibility of glucose utilization exists.

There is, however, in the blood no evidence of sugars other than glucose (fermentable sugar) and galactose, or of any reducing product formed from galactose. In Table II are shown the residual reducing substances of blood after the removal of fermentable sugar by yeast, and galactose by galac yeast. The remarkable constancy of this residual reducing fraction after the ingestion of either 40 or 80 gm. of galactose is ample evidence of our contention. Our figures also afford strong support to the conclusions of previous workers who have made the assumption that any increase in the non-fermentable fraction of blood could be reckoned as galactose under their experimental conditions.

*Blood Glucose*—Neither Corley (2) nor Cori and Cori (34) had been able to observe any glucose change after galactose feeding though Blanco (1) had noted very large increases in the well fed rabbit. Harding and van Nostrand (3) noted in a few of their subjects an undoubted increase in venous glucose following the administration of 50 gm. of galactose. Roe and Schwartzman's (4) figures show a reduction in blood glucose after the galactose ingestion in normal man. Their specimens were collected at 30, 60, and 120 minutes. Changes of greatest magnitude were to be found in the 60 minute specimen. In contrast to the fall in blood glucose in normals, they found a very marked increase over the same range of time if similar amounts of galactose were fed to diabetics.

Our present results on arterial blood show that the changes in blood glucose are varied in character. In a few instances there is little or no alteration. In most of our observations however there is a distinct rise, followed by a marked hypoglycemia. The rise is often only in the 15 or 30 minute sample. In a few instances, again, the hyperglycemia is of considerable magnitude (Table I). The arterial-venous blood glucose curves (Fig. 2) also illustrate the variety of results. The ephemeral nature of the hyperglycemia and the study of venous blood rather than arterial blood have

been mainly responsible for the conflicting results of previous investigators.

Does this transitory hyperglycemia represent a conversion of galactose to glucose? Impressed by the ability of man to retain galactose in the body (provided the dose was moderate), Harding and van Nostrand had been inclined to accept the occasional venous hyperglycemias as evidence of a direct galactose-glucose conversion, though they admitted the possibility of a galactose stimulus as the origin. The continued post-galactose hyperglycemia in diabetics has inclined Roe and Schwartzman to a belief in galactose-glucose conversion. While by no means denying the possibility of such a conversion, we now feel that no clear evidence has been obtained from a study of blood sugar changes. The irregular and transitory nature of the hyperglycemia, its independence of the amount of galactose intake (Fig. 1), the fact that similar transitory hyperglycemias can be noticed after the administration of other sugars where there is no physiological reason to assume a rapid conversion (35), all incline us to the belief that blood sugar studies, even if the changes are in the expected direction, form too unstable a basis to act as evidence. The blood sugar in diabetics is even more susceptible to exhibitions of hyperglycemia.

Our blood sugar studies, however, render it clear why no glucose has been found in normal urine after galactose ingestion. The hyperglycemias are much too small to allow of any escape through the kidney. In the diabetic the increased hyperglycemias might be expected to lead to extra urinary glucose.

*Urine Galactose*.—Our experiments offer conclusive evidence for the excretion of galactose in all subjects after galactose ingestion. Beyond the statement of Bodansky (27) of his inability to obtain mucic acid as evidence of urinary galactose after administration of that sugar to dogs, there has been no doubt in recent years of its excretion under similar experimental conditions. Halberkann and Kähler (36) have isolated the crystalline sugar from the urine of patients as a sequence to galactose tolerance tests.

Our experiments afford, however, not only further proof of the excretion of galactose, but evidence that there is no immediate excretion of any reducing metabolite other than galactose. In

Table III are shown the mg. of residual reducing substances for 2 hours preceding and 2 hours succeeding the administration. Harding and van Nostrand have shown the absence of any slow delayed excretion of galactose in the later hours. Only in a few cases is there any increase in the residual reducing substances. Such increases as we have observed are very small, and can well be due to experimental error. One error may be occasioned by the high dilution of the urine before the application of the analytical procedures, especially in urines with large amounts of galactose. Any failure to remove the last trace of galactose would give a large apparent increase in the residual reducing substances. Another possible error is incomplete collection of urine.

TABLE III

*Constancy of Residual Urinary Reducing Substances after Galactose*  
The results are expressed as glucose in mg. per 2 hours.

Subject	7 00 9 00 a m.	9 00 11 00 a m	Subject	7 00 9 00 a m	9 00-11 00 a m
R. F.	18	16	G. H.	42	48
G. B.	70	71	N. M.	24	24
B. C.	12	13	G. A. G.	40	40
W. F.	55	65	C. E. D.	26	24
R. G.	44	59	W. S.	33	41
J. S.	20	27	A. R. A.	24	22

This is more likely in the 7 to 9 a.m. specimen than in the 9 to 11 a.m. specimen. An incomplete control specimen would give a low figure for the residual reducing substances, resulting in an apparent increase in the second period. This latter error is very probable for we have noted that the best agreement between the residual reducing substances of the two experimental periods is found in the urines of subjects who are research workers, accustomed to metabolism experiments of this type and, therefore, very careful in the collection of urine specimens.

The possibility also exists that galactose might be excreted in a non-reducing form. As a partial inquiry into this possibility we have submitted the 7 to 9 and the 9 to 11 a.m. urines to hydrolysis by 0.5 N  $\text{H}_2\text{SO}_4$ . There is no evidence that any galactose is set free from a non-reducing compound by this procedure, nor

of the production of any large excess of reducing compounds in the 9 to 11 a.m. urine. These observations add weight to the argument of Harding and van Nostrand that galactose is a well utilized sugar.

Our experiments also supply further data on the question of a renal threshold for galactose. Folin and Berglund (26) had pointed out the practical non-existence of a threshold, on the basis that very small doses of galactose produced extra urinary reducing substances. Goldblatt (24) also observed the excretion of galactose without noticing any rise in blood sugar. Rowe and Chandler (14), however, maintained the presence of a renal threshold. Harding and van Nostrand (3) found excretory non-fermentable substances with little or no rise in venous blood sugar. The present results on arterial blood galactose and the 15 minute collection of samples make us positive that urinary galactose can occur with very little rise in blood galactose. In several of our experiments blood galactose does not rise over 10 mg. The smallest increase in blood galactose combined with its excretion occurred in Series D. Subject T. T. (not shown in Fig. 2), after the administration of 30 gm. of glucose + 40 gm. of galactose, had only 4 mg. of galactose per 100 cc. of arterial blood and the total 2 hour urinary excretion was 215 mg. If any renal threshold exists for galactose, it must be extremely low.

#### SUMMARY

A method is described for the estimation of glucose, galactose, and residual reducing substances in 0.5 cc. of cutaneous blood.

The limits of blood galactose are noted after the ingestion of 40 gm. of galactose.

The peak of blood galactose in such galactose tolerance tests is from 30 to 60 minutes (inclusive). At the end of 2 hours the blood galactose has reached zero, or is only a very few mg. per 100 cc. The height of the peak of blood galactose is not usually more than 30 mg. per 100 cc. but occasional increases to 60 mg. are found in normal men.

The excretion of urinary galactose varies up to 2 gm.

After ingestion of 80 gm. of galactose, both the height of the peak and the duration of the blood galactose are increased. Urinary galactose is also increased.

Distinct arterial-venous differences in blood galactose can be found.

Galactose tolerance tests at 4.00 p.m. instead of under the usual fasting morning conditions show no unusual variations, even when the noon meal is carbohydrate or fat.

The addition of 30 gm. of glucose to 40 gm. of galactose lowers the height of both the galactemia and the galactosuria. The hyperglycemia also seems less than would be expected after the ingestion of 30 gm. of glucose alone.

Rises in blood glucose are often noted after the ingestion of galactose. The hyperglycemia does not appear dependent on the amount of galactose intake. The rise in blood glucose affords no proof of a direct rapid galactose-glucose conversion.

There is no evidence of the immediate formation in blood or urine of any reducing substance as an intermediate galactose metabolite.

Further evidence for the non-existence of a renal threshold for galactose is given.

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## STUDIES ON CARBOHYDRATE METABOLISM

### I. THE INFLUENCE OF *d*-GLUCAL AND ITS DERIVATIVES, *d*-HYDROGLUCAL AND *d*-2-GLUCODESOSE, ON BLOOD SUGAR\*

By WERNER FREUDENBERG

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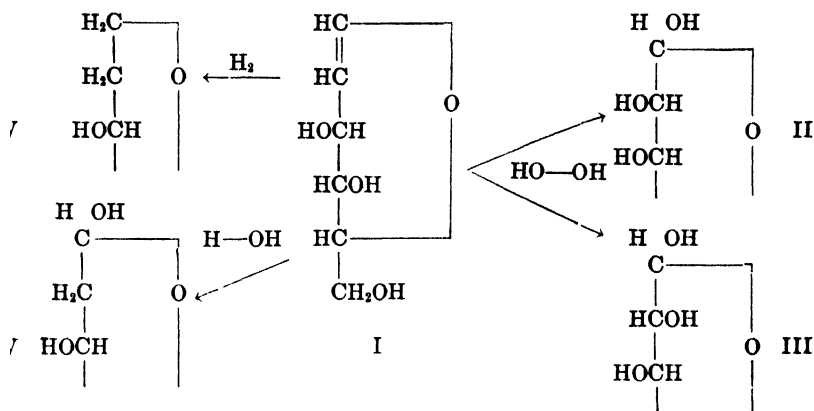
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Previous studies of carbohydrate metabolism have been limited almost exclusively to naturally occurring saturated sugars. Very little attention has been devoted to the fate of the unsaturated and anhydro sugars in the organism. Even though these unsaturated sugars occur very rarely in nature, information concerning their fate in the body would be of value in determining the mechanism of sugar metabolism.

Winter (1) reported that glucal injected into hypoglycemic rabbits relieves insulin convulsions, rebalancing the blood sugar level within a short period of time. The close chemical relation between glucal and 2-glucodesose led Winter (2) to a study of the physiological behavior of the latter substance. It proved, however, to be inactive. Kondo (3) showed that glucal after subcutaneous or oral administration was excreted by the rabbit in the urine in the form of 2-glucodesose. 2-Glucodesose itself after being given to rabbits was excreted unchanged. Only 2 to 3 per cent could be recovered from the urine as 2-glucodesose-*p*-nitrophenylhydrazone. With the exception of a study on the physiological action of  $\alpha$ -glucosan (4) no other work has been done on unsaturated and anhydro sugars in respect to their physiological significance.

The great chemical reactivity of glucal as a representative unsaturated sugar is illustrated by (I) to (V). Oxidation leads to *d*-mannose (II) and eventually to *d*-glucose (III); reduction to hydroglucal (IV); addition of water finally to 2-glucodesose (V).

\* This investigation has been aided by a grant from the Rockefeller Foundation to Iowa State College. Preliminary experiments were made while at the Department of Biochemistry, University of California, Berkeley.



(II) to (V) are abbreviated.

It is obvious that glucal offers more diversified reactions than does glucose. Very complicated reactions are necessary to isomerize glucose; oxidation and reduction yield sugar acids and alcohols which have lost their true sugar character and represent classes by themselves.

From these considerations, it seemed probable that in biological oxidation and reduction processes the use of unsaturated sugars might be of advantage, as compared to the saturated type, as a means of isolating recognizable intermediate or end-products. Whether or not the unsaturated sugars are metabolized can be decided by comparing the blood sugar data observed after administration, with similar data for closely related derivatives. If the possibility is excluded that the double bond of the unsaturated sugar is shifted by the organism into a different position,<sup>1</sup> it should be possible to draw conclusions about the structure and configuration of blood sugar. The chemical oxidation of glucal with perbenzoic acid yields 80 per cent of *d*-mannose and no *d*-glucose, its epimer (6). Since as a general rule (7) one of the epimers predominates, it should be possible to decide whether *d*-mannose or *d*-glucose is formed; both of them would be measured as blood sugar; *d*-mannose, however, could be isolated as its phenylhydrazone.

<sup>1</sup> Only one case is known in the chemistry of the glucals; namely, pseudoglucal (5).

EXPERIMENTAL<sup>2</sup>

*Methods*—The apparent blood sugar was determined by the method of Hagedorn and Jensen.<sup>3</sup> Considering Somogyi's investigations on the nature of blood sugar, it seemed essential also to obtain information concerning the changes of the fermentable or true sugar, and the reducing non-sugars (non-fermentable reducing substances). Fleischmann's yeast was found satisfactory for the fermentation of the *true sugar*; the values recorded represent the differences found between *apparent* and *reducing non-sugars*. The urine sugar determinations were performed by the Benedict method (9). The urine was collected in metabolism cages in which the rabbits were placed during the 24 hours preceding and following the experiments. Every blood sugar value recorded represents the average of at least three determinations.

The rabbits used were adult Himalayans. Their average body weight was approximately 2 kilos. These were kept during the experimental period on a diet consisting of 1 part of alfalfa and 1 part of ground oats. Food and water were given *ad libitum*. The animals were not fed for 24 hours from the morning preceding the day of the experiment.

The sugars in 15 per cent sterile water solutions were injected intravenously in the marginal vein of the ear. The dose of sugar was equal to 0.5 gm. per kilo of body weight. Special care was taken that the injection was completely intravenous, the experiments being discontinued if any of the solution accidentally entered the tissues.

*Preparation of Glucal, Hydroglucal, and 2-Glucodesose*—The compounds required were prepared according to the procedures of Bergmann and coworkers (6, 10) and Fischer (11). The constants

<sup>2</sup> The writer is greatly indebted to Eli Lilly and Company, Indianapolis, for the insulin used, and to Dr. C. H. Rayburn, for kindly contributing a greater part of the 2-glucodesose required.

<sup>3</sup> Recognition is made of the truth of Somogyi's criticism concerning the deproteinization in the Hagedorn-Jensen method (8), also that blood filtrates, prepared by this procedure give higher values when the sugar is determined with the ferricyanide reagent, than with the copper reagent. However, since the aim of this investigation is to detect differences in the blood sugars, it seemed justifiable to continue with the Hagedorn-Jensen method as initiated in preliminary experiments in order to retain experimental uniformity.

obtained are given in Table I. All rotations were determined in aqueous solution; the melting points are uncorrected.

*Effect of Glucal upon Blood Sugar*—When a solution of pure, crystalline glucal was injected intravenously into a normal fasting rabbit, the blood sugar rose slowly but steadily for 3 hours as recorded in Table II.

The fermentable sugar increases to approximately the same extent as the apparent sugar. The reducing non-sugars were only very slightly changed.

In hypoglycemic rabbits the intravenous injection of glucal caused not only speedy recovery from convulsions, but also a

TABLE I  
*Constants Obtained with Substances Used*

Compound	$[\alpha]_D^{20-24}$	M. p.
		°C.
Glucal	-7.03	56-58
	-7.10	57-59
2-Glucodesose	+46.0	147-148
	+45.5	148-149
Hydroglucal	+15.9	85-87

curtailment of the time required for the rebalancing of the normal level compared with that required after injection of glucose. This finding has been checked in four experiments on as many rabbits.

The results obtained by Winter (1) agree with the above observation. However, he did not emphasize the fact that the chemical activity of glucal as found and studied *in vitro*, is equally evident in its physiological behavior which was demonstrated in the shortened period of recovery.

Since there was no sign of a reducing sugar in the urine within the first 24 hours following injection of glucal, it seems unlikely that under these experimental conditions glucal is excreted in the urine in the form of 2-glucodesose as Kondo (3) found after oral and subcutaneous application of glucal. The reducing power of glucodesose is 80 per cent of that of glucose and any trace of glucodesose would be expected to give a distinct reduction.

That glucal could be oxidized to *d*-mannose, as found *in vitro*

by Bergmann and Schotte (6), did not appear to be applicable to the organism. In neither liver, blood filtrates, nor urine was the detection of *d*-mannose possible in spite of the extreme sensitivity of the phenylhydrazone reaction for this sugar.

*Physiological Action of Hydroglucal*—The expected physiological inactivity of hydroglucal, the hydrogenation product of glucal,

TABLE II  
*Effect of d-Glucal upon Blood Sugar*

Remarks	Time after injection		Blood sugar per 100 cc. blood, in terms of glucose		
			Apparent sugar	Fermentable sugar	Reducing non-sugars
	hrs.	min.	mg.	mg.	mg.
Rabbit 27 ♀, * weight 2200 gm. 1.15 gm. glucal	0	0	102	83	19
		15	120	88	32
		30	127	100	27
	1		135		
	2		147	122	25
	3		158	137	21
	5		105	81	24
Rabbit 21 ♂, * weight 2100 gm. 6 units insulin Convulsions 1.1 gm. glucal Appears normal	0	0	99	76	23
	2	10	38	19	19
		15	64	41	23
		30	83	64	19
	1		105	79	26
	1	30	110	86	24

\* The animals were not fed for 24 hours preceding the experiments.

was verified as shown by the results given in Table III. Neither apparent nor fermentable sugars nor reducing non-sugars were appreciably affected after hydroglucal injection. In hypoglycemic rabbits only very slow recovery took place; insulin convulsions could not be inhibited with hydroglucal. In no case after hydroglucal had been given was any sign of glycosuria found. However, it is not unlikely that the sugar was excreted unchanged.

Its isolation from the urine was not attempted in view of the small amounts given and the lack of a specific test for hydroglucal.

*Effect of 2-Glucodesose upon Blood Sugar*—Although the chemical transformation of glucal into 2-glucodesose involved merely the addition of the elements of water, there resulted a surprising

TABLE III  
*Effect of Hydroglucal upon Blood Sugar*

Remarks	Time after injection	Blood sugar per 100 cc. blood, in terms of glucose		
		Apparent sugar	Fermentable sugar	Reducing non-sugars
	<i>hrs.</i>	<i>min.</i>	<i>mg.</i>	<i>mg.</i>
Rabbit 22♂,* weight 2200 gm. 1.1 gm. hydroglucal	0	0	112	87
		15	120	98
		30	122	103
	1		125	99
	2		118	93
	3		120	98
	4		110	92
Rabbit 26♀,* weight 1900 gm. 5 units insulin 0.95 gm. hydroglucal	0	0	102	82
	3		32	16
		15	50	31
		30	48	26
	1		40	25
	2		64	43
	3		94	76
Weak through whole experiment				

\* The animals were not fed for 24 hours preceding the experiments.

change in the physiological activity as demonstrated in Table IV. Both apparent and fermentable sugar rose very rapidly for 3 hours following the injection. The increase in fermentable sugar showed that the whole reduction was not due to 2-glucodesose itself since this sugar is not fermentable with yeast. A more probable explanation is that liver glycogen was mobilized and reconverted into fermentable sugar. No explanation is offered for the high

values—37 mg. average—of the reducing non-sugars. The same grade of yeast was used as in previous experiments.

Despite Kondo's findings (3) there is no doubt that glucodesose is toxic. Injections of glucodesose greater than 0.5 gm. per kilo of body weight were in most cases lethal, presumably primarily

TABLE IV  
*Effect of 2-Glucodesose upon Blood Sugar*

Remarks	Time after injection		Blood sugar per 100 cc. blood, in terms of glucose		
			Apparent sugar	Fermentable sugar	Reducing non-sugars
	hrs.	min.	mg.	mg.	mg.
Rabbit 14 ♀, * weight 2070 gm. 1.0 gm. glucodesose	0	0	98	69	29
		15	146	113	33
		30	172	133	39
	1		207	171	36
	2		273	230	43
	3		283	244	39
	5		218	177	41
Rabbit 21 ♂, * weight 2100 gm. 5 units insulin 1.03 gm. glucodesose Convulsions " 0.5 gm. glucodesose Animal saved by injection of glucose	0	0	99	77	22
	2	30	38	21	17
		15	42	23	19
		30	45	25	20
	1		36	18	18
	1	10			
	1	20	40	18	22

\* The animals were not fed for 24 hours preceding the experiments.

affecting the lung and heart action. Death occurred within 5 to 10 minutes, accompanied by symptoms very similar to those resulting from lethal doses of adrenalin.

The injection of 2-glucodesose into a rabbit in insulin convulsions was without observable effect. This result confirms Winter's findings (2).

The substance was partially excreted unchanged by the rabbit as seen from a definite increase in reducing power in the urine, with Benedict's method for estimating the urine sugar. The increase amounted to 2.3 per cent in terms of glucose. Proof that the reduction was due to glucodesose could be furnished by isolating glucodesose from the urine in the form of its *p*-nitrophenylhydrazone. Kondo's procedure (3) has been used for the isolation.

#### DISCUSSION

The experiments reported above show that the organism is able to convert glugal primarily into blood sugar. If we assume that blood sugar is a normal glucose (glucopyranose), as now generally accepted (12), the oxidation of glugal in the body leads therefore to glucose, while as a result of chemical oxidation the epimeric form, mannose, entirely predominates.

The reactivity of glugal has been demonstrated in a biological process. The time of recovery from insulin convulsions in the rabbit is shorter after glugal administration compared to the time required by glucose. Hydroglugal and 2-glucodesose were found to be non-metabolizable. While the organism is able to utilize glugal by oxidizing it at the double bond, no point of attack is offered in its saturated derivatives, hydroglugal and 2-glucodesose.

#### SUMMARY

1. *d*-Glugal, the most completely described unsaturated sugar, is readily metabolized by the rabbit. In agreement with the findings of Winter it has been found to relieve convulsions caused by insulin.

2. Hydroglugal, the hydrogenation product of glugal, is physiologically inactive as found from blood sugar values under normal conditions and in animals previously treated with insulin.

3. 2-Glucodesose proved to be toxic when injected intravenously into rabbits. The lethal dose is greater than 0.5 gm. per kilo of body weight. 2-Glucodesose is unable to counteract insulin effects.

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## STUDIES ON CARBOHYDRATE METABOLISM

### II. THE RATE OF METABOLISM OF *d*-2-OXYGLUCAL AND STYRACITOL IN THE RABBIT\*

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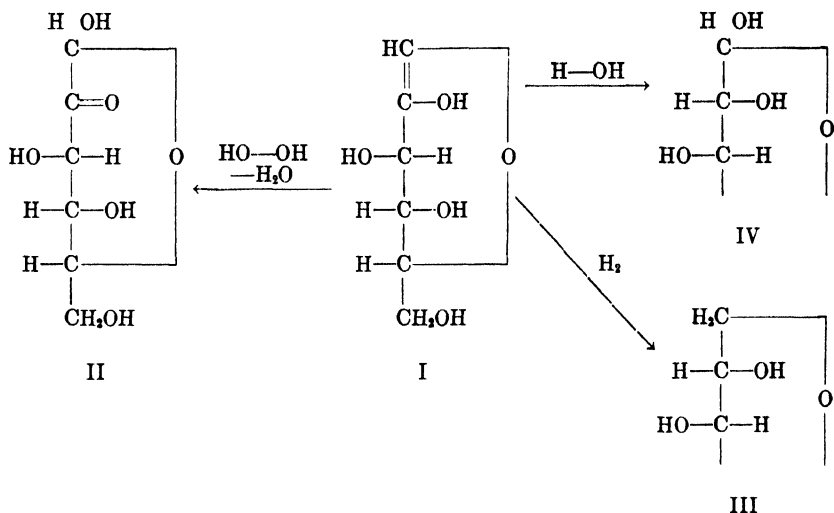
(Received for publication, September 12, 1932)

Thannhauser and Jenke (1) found that glucosone applied *per os* has a remarkable lowering effect upon the blood sugar level. Only its high toxicity prohibited the therapeutic use of this substance for diabetes treatment. Hynd (2) and Herring and Hynd (3) studying the action of glucosone confirmed Thannhauser's findings and concluded that glucosone is an important intermediate in the carbohydrate metabolism.

Since Fischer's discovery of glucosone, no investigator had succeeded in preparing the pure crystalline ketone-aldehyde, until recently Maurer and Petsch (4) in the course of studies on unsaturated sugars showed its relation to 2-oxyglucal, from which a synthesis of a crystalline acetyl derivative could be accomplished.

2-Oxyglucal (I), as shown by the formula, is a dehydrated hexose with a double bond between carbon atoms (1) and (2); the addition of a molecule of water to the double bond yields a saturated sugar of the type of glucose (IV). Oxidation and dehydration lead to glucosone (II). The catalytic reduction finally gives an alcohol, namely styracitol (III). It was isolated from a plant, *Styrax obassia*, native to Japan, by Ashina (5). Its relation to oxyglucal and its synthesis were reported in a recent article by Zervas (6).

\* This investigation has been aided by a grant from the Rockefeller Foundation to Iowa State College.



Reaction from (I) to (II) is according to Maurer and Petsch (4); (I) to (III), Zervas (6); (I) to (IV) is a theoretical reaction, reverse of the synthesis of 2-oxyglucal (Maurer (7)). (III) and (IV) are abbreviated.

A study of the physiological significance of 2-oxyglucal seemed desirable considering its close relationship to glucosone and glucose. It was possible that the biological oxidation of oxyglucal involved glucosone as an intermediate product of metabolism, and therefore would show similar physiological behavior. The examination of the action of styrcitol upon blood sugar would be essential to complete the knowledge of the physiological action of the sugar group mentioned, and to obtain data for comparing 2-oxyglucal with its reduction product. These experiments represent another attempt at the determination of the course of carbohydrate utilization in the blood stream where sugar structures with definite label groupings are used to map the course of the reaction.

#### EXPERIMENTAL<sup>1</sup>

*Methods*—The Hagedorn-Jensen method (8) was used for determining the *apparent* sugar, and the *true fermentable* sugar was

<sup>1</sup>We are obliged to Eli Lilly and Company for the insulin used in this investigation.

estimated according to Somogyi (9). The differences between apparent and fermentable sugars represent the values for the *reducing non-sugars*. Three complete experiments were made with each sugar. Since the results are identical, only one set of data is given in the experimental part. Each blood sugar value recorded represents the average of at least three determinations. The technique of injecting, the concentration of solutions used, the strain and diet of the rabbits are the same as described in the first communication (10).

TABLE I  
*Effect of 2-Oxyglucal upon Blood Sugar*

Remarks	Time after injection		Blood sugar per 100 cc. blood, in terms of glucose		
			Apparent sugar	Fermentable sugar	Reducing non-sugars
	hrs.	min.	mg.	mg.	mg.
Rabbit 11♂,* weight 2250 gm. 1.12 gm. oxyglucal	0	0	95	59	36
		15	157	114	43
		30	147	102	45
	1		116	74	42
	2		98	61	37
	2	30	98	57	41

\* The animal was not fed for 24 hours preceding the experiment.

*Preparation of 2-Oxyglucal*—The free 2-oxyglucal, in spite of various experiments of several investigators, has not yet been crystallized. A procedure given by Maurer (7) yields a product which is fairly pure. Only minor changes were made.

Tetraacetyloxyglucal was dissolved in methyl alcohol which had been previously saturated at 0° with ammonia. The solution was kept in a pressure bottle in the ice box for 24 hours. The solvent was then evaporated off *in vacuo* until the excess of ammonia present was quantitatively removed. The acetamide formed was separated by extracting with ethyl acetate. The residue was triturated successively with acetone, ether, and petroleum ether. A yellowish amorphous powder was obtained which still contained traces of nitrogen. It seemed identical with Maurer's

product, in spite of a slightly higher melting point (102°, not sharp). It decomposed when heated to 130–135°.

*Preparation of Styracitol*—The synthesis of styracitol was made according to Zervas (6). The product isolated corresponded exactly with his findings, with respect to physical properties such as melting point, rotation, and yield.

TABLE II  
*Effect of Styracitol upon Blood Sugar*

Remarks	Time after injection		Blood sugar per 100 cc. blood, in terms of glucose		
			Apparent sugar	Fermentable sugar	Reducing non-sugars
	hrs.	min.	mg.	mg.	mg.
Rabbit 4 ♂,* weight 2100 gm. 1.01 gm. styracitol	0	0	97	64	33
		15	105	69	36
		30	102	62	40
	1	15	98	65	33
	2		112	74	38
	3		102	65	37
Rabbit 13 ♂,* weight 1900 gm. 4 units insulin  0.97 gm. styracitol	0	0	130	94	36
	2		52	21	31
		15	57		
		30	60	35	25
	1		73		
	1	30	79	57	22
	3		93	64	29

\* The animals were not fed for 24 hours preceding the experiment.

*Effect of 2-Oxyglucal upon Blood Sugar*—Table I shows the changes in blood sugar after intravenous oxyglucal injection. The fact that fermentable sugar increases and decreases by approximately the same amount as the apparent sugar indicates that blood sugar has been measured and not oxyglucal; the latter does not reduce and is not fermentable. The values for reducing non-sugars are higher than those found in earlier experiments for

the same strain of rabbits. The rabbits were kept in metabolism cages and the urine before and after the experiments was collected and analyzed according to Benedict (11). No increase in reducing sugars could be found in the urine after the administration of 2-oxyglucal.

Contradictory results were obtained when 2-oxyglucal was given to hypoglycemic rabbits. The dose 0.5 gm. per kilo of body weight was insufficient for relieving convulsions caused by insulin (5 units). Larger amounts, however, 1 gm. per kilo of body weight, had a definite action upon animals in a less severe state of hypoglycemia (3 units of insulin).<sup>2</sup>

*Effect of Styracitol upon Blood Sugar*—The data recorded in Table II after intravenous injection of styracitol show clearly that this sugar alcohol is without effect upon blood sugar. Practically no rise is seen in apparent sugar; the reducing non-sugars remained approximately constant at 35 mg. per 100 cc. of blood in terms of glucose.

In hypoglycemic rabbits only a very slight increase in reducing sugars was found; the blood sugar level remained below normal for 3 hours after the injection. Insulin convulsions, therefore, could not be relieved by administering styracitol. In none of the experiments with styracitol was an increase of urine sugars (11) observed. No attempt was made to detect styracitol excreted in the urine, since only small amounts were used and a specific reaction for this sugar alcohol is lacking.

#### SUMMARY

1. An attempt has been made to prepare 2-oxyglucal in a pure form by hydrolyzing its tetraacetyl derivative by means of methyl alcohol saturated with ammonia.

2. 2-Oxyglucal as yet amorphous and still containing nitrogen causes a definite rise in apparent and fermentable blood sugar of rabbits. Contradictory results, however, were obtained when 2-oxyglucal was given to hypoglycemic rabbits. The injection of

<sup>2</sup> It seems unsafe to draw definite conclusions from these experiments. The question of whether or not 2-oxyglucal is metabolized must be decided by other means such as determining the rate of glycogen formation or by using for instance Cori's method (12) of estimating the extent of absorption from the intestinal tract.

oxyglucal is not followed by glycosuria. It is concluded that 2-oxyglucal in the form used is only partially metabolized. The rise in blood sugar after oxyglucal administration proves that no glucosone is formed by the body, since glucosone has a strong lowering effect upon the blood sugar level.

3. Styrcitol, the hydrogenation product of 2-oxyglucal, is physiologically inactive.

4. A further example has been given to show that the organism is able to transform unsaturated sugars with a double bond between carbon atoms (1) and (2) into blood sugar. Saturation, hydrogenation in the example given, eliminates the physiological effect of the parent substance.

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# THE FREE ENERGIES OF FORMATION OF AQUEOUS *d*-ALANINE, *l*-ASPARTIC ACID, AND *d*-GLUTAMIC ACID

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The employment of thermodynamics in biochemistry has been restricted, until recently, to the use of first law data. In the last few years a beginning has been made in the application of the second law; *i.e.*, of free energy data (1, 2). The development of this field is limited by the paucity of available free energy data. We have therefore undertaken the systematic determination of the free energies of formation of compounds which may be interesting in biochemistry or physiology.

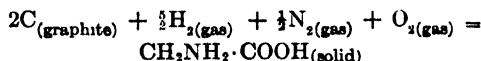
At the outset the query is met, of course, as to the universal validity of the second law of thermodynamics in biological systems. We shall deal with the general question in future publications. For the present it is profitable to assume that organisms obey the second law of thermodynamics as do macroscopic systems.

We shall assume, therefore, that the theoretical maximum amount of work derivable from any process is given by the equation<sup>1</sup>

$$w_R = -\Delta F + \Delta(PV) \quad (1)$$

where  $w_R$  is the work performed when the reaction is carried out in a perfectly reversible manner,  $-\Delta F$  the difference in free energy between initial and final states (*i.e.*  $F_1 - F_2$ ), and  $\Delta(PV)$  the change in the pressure-volume product,  $P_2V_2 - P_1V_1$ . Further in any spontaneous process  $-\Delta F$  is a positive quantity.

Let us consider the hypothetical reaction



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<sup>1</sup> The symbols used throughout are those of Lewis and Randall (3).

which is the equation for the formation of glycine from the elements. The free energy change for this reaction<sup>†</sup> is given by the equation

$$\Delta F_{298} = \Delta H - T\Delta S \quad (2)$$

We can evaluate  $\Delta F$  if  $\Delta H$  and  $\Delta S$  are known at 25°.

$\Delta H_{298}$  may be obtained from the value of the heat of combustion at constant pressure and at 25°. It is simply the sum of the heat of combustion and the known heats of formation of the number of mols of water and carbon dioxide that are formed. This may be written in the form of the general equation

$$\Delta H = \text{heat of combustion} + a\Delta H_{\text{H}_2\text{O}} + b\Delta H_{\text{CO}_2} \quad (3)$$

where  $a$ ,  $b$ , etc., represent the number of mols of the substance taken.

In a like manner  $\Delta S_{298}$  is the difference between  $S_{298}$  of the compound and the corresponding known entropies of the elements. This may be written

$$\Delta S_{298} = S_{(\text{compound}, 298)} - aS_{(\text{C}, 298)} - bS_{(\text{H}_2, 298)} - cS_{(\text{O}_2, 298)} - dS_{(\text{N}_2, 298)} \quad (4)$$

If we accept the third law of thermodynamics, the entropy of the compound may be determined from the relation

$$S_{298} = \int_0^T \frac{C_p}{T} dT + \frac{\Delta H}{T} + \int_T^{298} \frac{C_p}{T} dT \quad (5)$$

This integral requires that the heat capacity and any heats of transition be known over the complete temperature range.

In our work we have determined  $C_p$  and any transitions from the temperature of liquid air to that of the room. Consequently we can evaluate the portion of the integral between 90–298°K. graphically. For the portion of the integral between 0–90°K. we have used the empirical extrapolation formula of Kelley, Parks, and Huffman (4). This method is based on actual experimental heat capacity measurements to very low temperatures.

The values we have used for the heats of formation of water and carbon dioxide and the values for the entropies of the elements are given in Table I.

It is obvious that the value of the free energy of formation of a

substance in its pure crystalline or liquid state will rarely be useful as such in physiological calculations. Most substances *in vivo* are in dilute solution, and as an additional complication, the hydrogen ion concentration varies with different experimental conditions, and from species to species. Further, at different times the substances under consideration are weak acids or bases, amphoteric electrolytes, or neutral compounds. In view of these considerations, the selection of one standard reference state for all compounds seems inadvisable. We shall, in preference, employ different standard states according to the substance and the case under consideration.

### *d*-Alanine

Because of their bearing on several problems in nitrogen metabolism, we have begun with the determination of the free energies

TABLE I  
Heats of Formation and Entropies of Elementary Data

Heats of formation			Entropies of elements		
Substance	$\Delta H_{298}$	Bibliographic No.	Element	$S_{298}$	Bibliographic No.
H <sub>2</sub> O(liquid)	-68,310	5	C(graphite)	1.3	7
CO <sub>2</sub> (gas)	-94,240	6	H <sub>2</sub> (gas)	31.23	8
			N <sub>2</sub> (gas)	45.78	9
			O <sub>2</sub> (gas)	49.03	10

of *d*-alanine, *d*-glutamic acid, and *l*-aspartic acid. As an example of the way in which the heat capacity measurements are used in these calculations, experimental values for *d*-alanine are given in Table II. The combination of the graphical integration of these heat capacity data between 90–298°K. with the value determined by extrapolation from 90–0°K. gives the value for  $S_{298}$  of 31.6 E.U. This value combined with the  $S_{298}$  values for the elements (Table I) gives a value of -153.5 E.U. for the  $\Delta S_{298}$  of formation.

The heat of combustion of *d*-alanine at constant pressure and corrected to 25° is 387,200 calories. When this value is combined with the values for the heats of formation of water and carbon dioxide (Table I)  $\Delta H$  is -134,600 calories. If these values are substituted in Equation 2,  $\Delta F_{298} = -134,600 - (298.1)(-153.5) = -88,850$  calories.

In order to compute the free energy of a substance in solution

its solubility and corresponding activity coefficient must be known. By interpolation from the data given by Seidell (11), the solubility of *d*-alanine at 25° is 16.7 gm. in 100 gm. of water. According to Frankel (12) the activity coefficient of *d*-alanine in water at concentrations as high as 13.4 gm. in 100 gm. of water is 1. Freezing point depression and refractometric measurements at 30° gave the same result.

The equation for the concentration of the *Zwitter Ion* form is

$$\text{alanine} = A \left( 1 + \frac{(H^+)}{K_A} + \frac{K_W}{B \cdot (H^+)} \right) \quad (6)$$

The values for  $pK_A$  and  $(pK_W - pK_B)$  at 25° are 2.34 and 9.69 respectively (13). The hydrogen ion concentration of the satu-

TABLE II  
*Specific Heats at Constant Pressure of Crystalline d-Alanine*

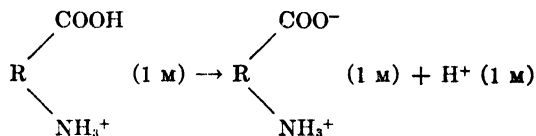
Temperature	Specific heats at constant pressure $C_p$	Temperature	Specific heats at constant pressure $C_p$
°K.	calories per gm.	°K.	calories per gm.
84.4	0.121	179.9	0.222
89.2	0.127	200.1	0.241
96.2	0.135	219.9	0.258
104.2	0.146	239.8	0.275
117.2	0.154	260.0	0.292
119.5	0.163	275.1	0.306
134.6	0.180	276.3	0.306
149.2	0.194	281.2	0.310
165.4	0.209	289.6	0.318
		296.8	0.324

rated solution of alanine at 25° computed by means of the equation of Sørensen (14) is  $8.1 \times 10^{-7}$ . Insertion of these values in Equation 6 shows that all but a negligible quantity of the ampholyte is in the *Zwitter Ion* form.

The free energy change in the transfer of alanine from its saturated solution to a 1 M solution is

$$\begin{aligned} -\Delta F_{298} &= RT \ln 167/89.1 \\ &= (1.989) (298.1) (2.303) \log 167/89.1 = 370 \text{ calories} \\ \therefore \Delta F_{(\text{saturated solution})_{298}} - \Delta F_{(1 \text{ M})_{298}} &= 370 \text{ calories} \\ \Delta F_{(1 \text{ M})_{25}} - 88,850 - 370 &= -89,220 \text{ calories} \end{aligned}$$

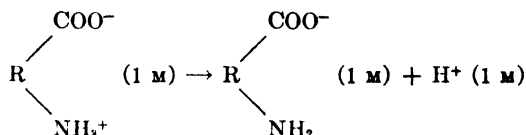
The free energies of the two ions may be computed as follows:



$$-\Delta F = RT \ln K_A = (-1365)(2.34) = -3190 \text{ calories}$$

$$\therefore \Delta F_{(\text{cation}, 1\text{ M})_{298}} = -92,410 \text{ calories}$$

Similarly for the basic dissociation,



$$-\Delta F = RT \ln \frac{K_W}{K_B} = -1365 (\text{p}K_W - \text{p}K_B) = (-1365)(9.69)$$

$$= -13,230 \text{ calories}$$

$$\therefore \Delta F_{(\text{anion}, 1\text{ M})_{298}} = -89,220 + 13,230 = -75,990 \text{ calories}$$

The constants employed in the calculation of the free energy changes in ionization are not strictly the thermodynamic dissociation constants. They are titration or apparent dissociation constants. These two types of constants are related to each other as follows:

$$K' = (\text{H}^+) \frac{(\text{A}^-)}{(\text{HA})} = \text{titration constant}$$

$$K = (\text{H}^+) \frac{(\text{A}^-)\gamma_-}{(\text{HA})\gamma_u} = \text{thermodynamic dissociation constant}$$

where  $\gamma_u$  denotes the activity coefficient of the undissociated acid.

On conversion to negative logarithms  $\text{p}K = \text{p}K' - \log \frac{\gamma_-}{\gamma_u}$ .

Simms (15) found that within the range of ionic strength from 0.05 to 0.3 the value of  $\text{p}K'$  for glycine was practically constant.

The activity coefficient ratio is therefore 1, and we may consider in the case of alanine as well as glycine  $pK = pK'$ .

*l*-Aspartic Acid

From values published elsewhere (16), the thermal data for *l*-aspartic acid are:

$$S_{298} = 41.5 \text{ E.U.}, \Delta S_{298} = -193.9 \text{ E.U.}$$

$$\Delta H_{298} = -231,300 \text{ calories}, \Delta F_{298} = -173,500 \text{ calories}$$

The solubility of *l*-aspartic acid at 25° is  $3.63 \times 10^{-2}$  mols in 1000 gm. of water (17). Because of the insufficiency of data regarding the activity coefficients of aspartic acid and its ions in the saturated solution, the computation of the free energy of solution can, at present, be only approximate. Two methods are open: One is the extrapolation of the calculations of Hoskins, Randall, and Schmidt (18) of the activity coefficients of aspartic acid to the saturated solution; the other is to calculate the degree of ionization of the aspartic acid in the saturated solution after computing the hydrogen ion concentration of the saturated solution at 25° by means of the equation of Sørensen (14), the titration constants at this temperature being employed. The value obtained for the concentration of the neutral aspartic acid by the second method may be taken, as an approximation, as equal to its activity (since titration constants and not the true dissociation constants were used in computing the pH of the saturated solution, and also the degree of dissociation). We have preferred the second method chiefly because of the uncertainty of the necessary double extrapolations of the activity coefficients to both a higher concentration and a higher temperature. The extrapolation of the activity coefficient from the freezing point to 25° is particularly uncertain because it is probable that with aspartic acid, on account of the great tendency of its neutral molecules to form aggregates, there is a considerable heat of dilution, which would make the activity coefficient at 25° quite different from that at the freezing point.

We have, nevertheless, computed the free energy of solution by both methods and have found the difference to be 50 calories. The details of only the second method are given. The values of the titration constants at 25° are  $pK_{A_1} = 1.90$ ;  $pK_{A_2} = 3.63$ ;

$(\text{p}K_W - \text{p}K_B) = 9.47$  (13). These yield a value for the hydrogen ion concentration in the saturated solution at  $25^\circ$  of  $5.5 \times 10^{-4}$ . The degree of ionization of aspartic acid is given by the equation

$$\Sigma \text{ aspartic acid} = A^\pm \left[ 1 + \frac{(\text{H}^+)}{K_{A_1}} + \frac{K_{A_2}}{(\text{H}^+)} + \frac{K_W}{K_B} \cdot \frac{K_{A_2}}{(\text{H}^+)} \right] \quad (7)$$

When the above values are inserted in this equation,  $A^\pm = 0.72$ .

The free energy of solution at  $25^\circ$  is  $RT \ln (0.036) (0.72) = -2160$  calories.

The free energy of the neutral molecule at 1 M activity is  $\Delta F_{(A^\pm, 1 \text{ M})_{298}} = -173,500 + 2160 = -171,340$  calories.

The free energies of the ions of aspartic acid are, as in the case of alanine, as follows:

$$A^+ (1 \text{ M}) = A^\pm (1 \text{ M}) + \text{H}^+ (1 \text{ M})$$

$$-\Delta F = RT \ln K_{A_1} = -2590 \text{ calories, } \therefore \Delta F_{(A^+, 1 \text{ M})_{298}} = -173,930 \text{ calories}$$

$$A^\pm (1 \text{ M}) = A^\pm (1 \text{ M}) + \text{H}^+ (1 \text{ M})$$

$$-\Delta F = RT \ln K_{A_2} = -4950 \text{ calories, } \therefore \Delta F_{(A^\pm, 1 \text{ M})_{298}} = -166,390 \text{ calories}$$

$$A^\pm (1 \text{ M}) = A^\pm (1 \text{ M}) + \text{H}^+ (1 \text{ M})$$

$$-\Delta F = RT \ln \frac{K_W}{K_B} = -12,930 \text{ calories, } \therefore \Delta F_{(A^\pm, 1 \text{ M})_{298}} = -153,460 \text{ calories}$$

#### *d*-Glutamic Acid

The thermal data for *d*-glutamic acid are

$$S_{298} = 45.7 \text{ E.U., } \Delta S_{298} = -222.3 \text{ E.U.}$$

$$\Delta H_{298} = -236,400 \text{ calories, } \Delta F_{298} = -170,200 \text{ calories}$$

The solubility at  $25^\circ$  is 0.058 mol in 1000 gm. of water (17). The free energy of solution was calculated by the method which was preferred and set out in detail for aspartic acid. The titration constants at  $25^\circ$  are  $\text{p}K_{A_1} = 2.10$ ,  $\text{p}K_{A_2} = 4.07$ , and  $(\text{p}K_W - \text{p}K_B) = 9.47$  (13). The hydrogen ion concentration of the saturated solution at  $25^\circ$  calculated by the method of Sørensen, is  $7.7 \times 10^{-4}$ . Insertion of these values into Equation 7 gives 83 per cent of the total glutamic acid in the neutral form in a saturated solution.

The free energy of solution therefore is  $RT \ln (0.83) (0.058) = -1800$  calories. The value obtained by extrapolating from the data of Hoskins, Randall, and Schmidt is  $-1890$  calories. If the first value is taken for the free energy of solution, the free energy of neutral glutamic acid at 1 M activity is  $-168,400$  calories. The free energies of the ions at  $25^\circ$  computed as in the case of aspartic acid, by the use of titration constants, are:

$$G^+ (1 \text{ M}) = G^\pm (1 \text{ M}) + H^+ (1 \text{ M})$$

$$-\Delta F = RT \ln K_{A_1} = -2870 \text{ calories, } \therefore \Delta F_{(G^+, 1 \text{ M})_{298}} = -171,270 \text{ calories}$$

$$G^\pm (1 \text{ M}) = G^\pm (1 \text{ M}) + H^+ (1 \text{ M})$$

$$-\Delta F = RT \ln K_{A_2} = -5560 \text{ calories, } \therefore \Delta F_{(G^\pm, 1 \text{ M})_{298}} = -162,840 \text{ calories}$$

$$G^\pm (1 \text{ M}) = G^\equiv (1 \text{ M}) + H^+ (1 \text{ M})$$

$$-\Delta F = RT \ln \frac{K_W}{K_B} = -12,930 \text{ calories, } \therefore \Delta F_{(G^\equiv, 1 \text{ M})_{298}} = -149,910 \text{ calories}$$

The rounded off values of the free energies of the different species of the three amino acids are collected in Table III.

#### *l*-Aspartate-Enzyme-Fumarate-Ammonium Equilibrium

Quastel and Woolf (19) observed that in the presence of *Bacillus coli communis* treated with growth inhibitors such as toluene, sodium nitrite, or propyl alcohol, an equilibrium is attained between *l*-aspartic acid, ammonia, and fumaric acid. Cook and Woolf (20) confirmed these results and extended the observations to other organisms. Woolf (21) found later that in the earlier formulation of the equilibrium they had overlooked the equilibrium between fumaric and *l*-malic acids. With this correction the value of the equilibrium constant  $\frac{(\text{fumaric acid})(\text{ammonia})}{(\text{l-aspartic acid})}$  becomes approximately 0.01 at  $37^\circ$ .

With the determination of the free energy of *l*-aspartic acid, it became possible to compare this experimentally obtained equilibrium constant with that which can be computed from purely thermal data; i.e., in which the intervention of the enzyme is ignored. Such a comparison of calculated and observed equilibrium constants was made in the case of the succinate-enzyme-

fumarate equilibrium. In that case it was found that the enzyme might be considered as a perfect catalyst (2).

On account of the complexity of the details in computing equilibria in solution from thermal data the following outline of the general plan is presented.

1. The equilibrium was measured at pH 7.4 and at 37°. It is necessary, therefore, to compute the free energies of the participants in the reaction at 37° from the given data at 25°.

(a) Reliable data regarding the heats of solution and of dilution of *l*-aspartic acid are not yet available. Accordingly, we have com-

TABLE III  
*Free Energies of Different Species of Three Amino Acids at 25°*

Amino acid	Free energies of				
	Crystalline	Neutral (1 M)	Mono-valent cation (1 M)	Mono-valent anion (1 M)	Divalent anion (1 M)
	calories	calories	calories	calories	calories
<i>d</i> -Alanine.....	-88,850	-89,200	-92,400	-76,000	
<i>l</i> -Aspartic acid.....	-173,500	-171,300	-173,900	-166,400	-153,450
<i>d</i> -Glutamic ".....	-170,200	-168,400	-171,250	-162,850	-149,900

puted the free energy of crystalline *l*-aspartic acid at 37° by means of the equation

$$\frac{\Delta F_2}{T_2} - \frac{\Delta F_1}{T_1} = \int_{T_1}^{T_2} -\frac{\Delta H}{T^2} dT \quad (8)$$

Over the short temperature range of 12°  $\Delta H$  may be taken as constant with a negligible error.

(b) Calculation of the free energy of solution at 37° from the solubility and degree of ionization at this temperature.

(c) Calculation of the free energy of the monovalent anion of *l*-aspartic acid from the free energy of the neutral molecule in solution and the dissociation constant at 37°.

(d, e) Similar calculations of the free energies of the bivalent fumarate ion and of the ammonium ion at 37°. At the pH at which the reaction is carried out we may consider all of the fumaric acid to be in the form of the bivalent ion, the ammonia as ammonium ion, and the *l*-aspartic acid as the monovalent anion.

2. Definition of the thermodynamic environment. (a) Computation of the ionic strength from the composition of the solution; (b) estimation of the activities of the various components of the reaction.

3. (a) Conversion of the given molal equilibrium concentrations into their corresponding activities with the corresponding modification of the value of the equilibrium constant.

(b) Calculation of the free energy change corresponding to this modified equilibrium constant by means of the equation  $-\Delta F = RT \ln K$ .

(c) Calculation of  $-\Delta F$  from the values in (1, c and d), derived from the thermal data, by means of the equation

$$-\Delta F(\text{reaction}) = \Delta F(\text{aspartate}^{\pm}, 1 \text{ M}) - \Delta F(\text{fumarate}^{\pm}, 1 \text{ M}) \\ - \Delta F(\text{NH}_4^+, 1 \text{ M})$$

1. (a) *l-Aspartic Acid*

$$\Delta F_{(\text{solid})_{298}} = -173,500, \Delta H_{298} = -231,300, \Delta F_{(\text{solid})_{310}} = -171,175 \text{ calories}$$

(b) The solubility of *l*-aspartic acid at  $37^\circ$  is  $5.5 \times 10^{-2}$  mols per 1000 gm. of water. From the data given by Cohn (13) the ionization constants at  $37^\circ$  are  $\text{p}K_{A1} = 1.85$ ,  $\text{p}K_{A2} = 3.57$ , and  $(\text{p}K_W - \text{p}K_B) = 9.12$ . The hydrogen ion concentration of the saturated solution is  $1.7 \times 10^{-3}$ . The fraction of the total aspartic acid in the neutral form according to the above values is 78 per cent. The free energy of solution therefore is  $RT \ln (0.055) (0.78) = -1940$  calories. The free energy of the neutral molecule at 1 M activity therefore is  $-171,175 + 1940 = -169,250$  calories.

(c) The free energy of the monovalent aspartate ion is given by the equation

$$A^{\pm} (1 \text{ M}) = A^{\pm} (1 \text{ M}) + \text{H}^+ (1 \text{ M})$$

$$-\Delta F = RT \ln K_{A_1} = -5050 \text{ calories}, \therefore \Delta F_{(A^{\pm}, 1 \text{ M})_{310}} = -164,200 \text{ calories}$$

(d) *Fumaric Acid*—We have previously calculated (2) the free energy of the bivalent fumarate ion at 1 M activity to be  $-144,630$  calories, and its heat of formation as  $-189,120$  calories. Hence the free energy of the bivalent fumarate ion at  $37^\circ$  is  $-142,850$  calories.

(e) *Ammonium Ion*—The free energy of  $\text{NH}_4^+$  at 298°K. is given by Lewis and Randall (3) as  $-18,930$  calories and the heat content as  $-31,790$ . Hence the free energy of this ion at 37° is  $-18,400$  calories.

2. (a) The ionic strength in one of the experiments quoted by Woolf was 0.22. (b) It is not possible from purely theoretical considerations to compute the precise activity coefficients of any of the participants in this reaction in solutions whose ionic strengths are as great as this. The available experimental data guide us only to a reasonable guess. From the following simplified form of the Debye-Hückel equation

$$-\log \gamma_i = \frac{0.5 z_i^2 \sqrt{\mu}}{1 + 3.3 \times 10^7 a \sqrt{\mu}} \quad (9)$$

where  $\gamma_i$  is the activity coefficient of the ions of the  $i$ th kind,  $z$  its valency,  $a$  is a constant taken as  $10^{-8}$ , and  $\mu$  the ionic strength, the activity coefficients of the ammonium ion, bivalent fumarate ion, and aspartate ion are 0.63, 0.15, and 0.63 respectively. The table given by Lewis and Randall ((3) p. 382) suggests that, if the ammonium ion resembles  $\text{Na}^+$ , or  $\text{K}^+$ , 0.7 is probably nearer the true value for its activity coefficient.

A value of the order of magnitude of 0.7 for the activity coefficient of the aspartate ion is similarly in accord with an extrapolation of the data of Hoskins, Randall, and Schmidt of the activity coefficient of monosodium aspartate. The measurements of Simms indicate that 3.3 instead of 4 is a more justifiable figure for  $z_i^2$  for the bivalent fumarate ion on account of the distance between the charges. This gives a value of 0.215 for the activity coefficient of this ion. At the pH of the experiment, approximately 7.35, 97 per cent of the ammonia is in the form of ammonium ion. In view of other uncertainties this correction may be neglected. All the fumaric acid may be taken as bivalent fumarate ion and the aspartic acid as the monovalent aspartate ion.

We are now in a position to attempt an approximate evaluation of the equilibrium constant. In the experiment which has been referred to above, the initial concentrations of fumaric acid and ammonia were 0.1 M. At equilibrium 28 per cent of the initial quantity of ammonia was found. The remainder had combined

with an equivalent amount of fumaric acid to form *l*-aspartic acid. The chemical reaction may be written  $l\text{-aspartate}^{\pm} = \text{fumarate}^{-} + \text{NH}_4^{+}$ .

The mass law expression, if activity coefficients are ignored, is 
$$\frac{(0.028)(0.028)}{(0.072)} = 0.011.$$

The free energy change is  $-\Delta F = RT \ln 0.011 = -2800$  calories.

When the activity coefficients are taken into account, the equilibrium expression is 
$$\frac{(0.028)(0.7)(0.028)(0.22)}{(0.072)(0.7)} = 0.0024.$$

The free energy change is  $-\Delta F = RT \ln 0.0024 = -3700$  calories.

TABLE IV  
*Comparison of Free Energy Values Obtained from Thermal and Equilibrium Data for Reaction at 37°*

$l\text{-Aspartate}^{\pm} = \text{fumarate}^{-} + \text{NH}_4^{+}.$

Quantity	Free energy values calories
$\Delta F$ ( <i>l</i> -aspartate $^{\pm}$ )	-166,000
	-164,200
	-163,500
$\Delta F$ (fumarate $^{-}$ )	-142,850
$\Delta F$ (NH $_4^{+}$ )	-18,400
$-\Delta F$ (reaction) from thermal data	-4,750
	-2,950
	-2,250
$-\Delta F = RT \ln \frac{(C_1)(C_2)}{(C_3)}$	-2,800
$-\Delta F = RT \ln \frac{(C_{1\gamma_1})(C_{2\gamma_2})}{(C_{3\gamma_3})}$	-3,700

From the thermal data, by using the heat of combustion as given in the International Critical Tables (22) for aspartic acid, we find for the reaction  $-\Delta F_{310} = -164,200 + 142,850 + 18,400 = -2950$  calories.

From the values of Emery and Benedict (23) and of Fischer and Wrede (24) for the heat of combustion of *l*-aspartic acid two other values for  $-\Delta F$ , namely -4750 and -2250, are obtained.

The observed value therefore agrees with that calculated within the limits of accuracy of the available thermal data. The con-

clusion is warranted, therefore, that here, as in the case of the succinate-fumarate equilibrium, the enzyme operates as a perfect catalyst. Stated in another form, we may conclude that the free energy values obtained from third law data may be used in the consideration of this reaction in metabolism, without taking into account the intervention of the enzyme.

The results of the above computations are collected in Table IV.

#### SUMMARY

1. The entropies, heats, and free energies of formation of *d*-alanine, *l*-aspartic acid, and *d*-glutamic acid, and their ions, at 25° are presented.

2. The free energy change in the reaction  $l\text{-aspartate}^{\pm} = \text{fumarate}^{-} + \text{NH}_4^{+}$  is computed from thermal data.

3. This value is shown to be in agreement with that calculated from the observed equilibrium effected by microorganisms, within the limits of accuracy of the thermal data.

4. It is concluded that the enzyme in this reaction operates as a perfect catalyst.

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## STUDIES ON ARGININE

### III. THE ARGININE CONTENT OF VERTEBRATE AND INVERTEBRATE MUSCLE\*

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#### INTRODUCTION

The investigations to be reported here were undertaken as a comparative study of the distribution of arginine in various animal species.

The foundation for such a study is to be found in the extensive contributions of Kutscher, Ackermann, and their associates on the nitrogenous bases of muscle, and in the more recent work of Meyerhof and Lohmann which gives to arginine a rôle of considerable importance in invertebrate muscle. From a summary published in 1926 (1) Kutscher and Ackermann were able to conclude that arginine, though a characteristic constituent of invertebrate muscle, was probably absent from the vertebrates in which, presumably, it is replaced by creatine. Since these investigations were qualitative in nature, and the conclusions dependent upon the direct isolation of arginine, we felt that a quantitative study by micro methods might reveal the presence of small amounts of arginine which would otherwise escape detection. Here it should be mentioned, that Abderhalden, in 1913 (2), had reported the isolation of free arginine from the blood of slaughterhouse animals. Weber (3) used a modified form of Sakaguchi's hypobromite method and demonstrated its presence in dog blood; while Kiech, Luck, and Smith (4), using an arginase-xanthhydrol method, reported its presence in rat muscle and liver. It is, however, absent from bovine spleen (5). Since Kutscher and

\* The studies on vertebrate muscle were made by Audra Arnold; those on invertebrates, by J. M. Luck.

Ackermann (6) have recently attempted the isolation of arginine from horse muscle, again with negative results, we considered it of value to analyze the muscles of other vertebrates, in the expectation that the arginine content of some species would be inappreciable.

As for invertebrates, we sought to determine, primarily, the total non-protein arginine (free arginine + phosphoarginine) content of muscle. Occasionally other organs were analyzed. The question of greatest interest was whether phosphoarginine is the only phosphagen in invertebrates or whether other phosphagens, restricted to certain phyla, exist. Eggleton and Eggleton (7) were able to show that phosphocreatine is present in none of the invertebrates examined by them. In the Crustacea, phosphocreatine is replaced by phosphoarginine (8). According to Meyerhof (9) this is also true of the molluscs, echinoderms, and sipunculids. Although Meyerhof's conclusions rest upon the determination of labile phosphorus in but a few species, they are supported by several arginine determinations as well. The recent work of Needham, Needham, Baldwin, and Yudkin (10)<sup>1</sup> on the phosphagen content of invertebrates is more comprehensive. Numerous species were examined and both phosphocreatine and phosphoarginine were determined. In some cases, however, there is room for doubt as to whether the more stable form of phosphagen, provisionally regarded by them as phosphoarginine, was really this compound,—a question to which we shall presently return.

#### *Analytical Methods*

The method used for determining arginine was essentially that of Kiech, Luck, and Smith (4). We soon discovered, however, that the procedure used by them for clarifying the protein-free tissue extracts was unsatisfactory and much attention was given to improving this step in the procedure. The use of copper sulfate and barium hydroxide as prescribed in the original method gave filtrates which were clear and sparkling, but which were frequently light blue in color and gave on addition of glacial acetic acid, with xanthidrol, a faint cloud of precipitate. The precipitate formed very slowly and was usually almost imperceptible; nevertheless,

<sup>1</sup> Cf. also the interesting paper by Needham and Needham (11).

it was sufficient to contaminate the dioxanthidryl urea which forms on addition of xanthidrol. In extreme cases the dry residue of dioxanthidryl urea instead of being white with a pearly luster, would be slightly colored and difficultly soluble in sulfuric acid. Examination of some of the material precipitated from a tungstic acid extract of muscle with glacial acetic acid (no clarifying agent being added) showed it to be a mixture of tungstates, containing 4 per cent of nitrogen. The ash was 87 per cent tungsten. Taking advantage of the very low solubility of barium tungstate in a slightly acid medium, we found that complete clarification was easily achieved by the use of barium acetate in place of copper sulfate and barium hydroxide. The filtrates now obtained were sparkling and colorless and in no case gave the slightest precipitate on treatment with an equal volume of glacial acetic acid. When xanthidrol was subsequently added the dioxanthidryl urea which separated out was crystalline, lustrous, and characteristic in all respects of pure dioxanthidryl urea. Before this form of clarification was developed, we tried various adsorbents, other protein precipitants, the salts of heavy metals, alkaloidal reagents, and barium salts at various acidities. We are now convinced that barium acetate as used in these studies serves perfectly in the clarification of tungstic acid extracts for we have employed it with uniform success in several hundred determinations on many different species.

Other steps in the analysis require mention.

*Preparation of Tissue Sample*—For the determination of total non-protein arginine the use of liquid air is to be recommended. It aids greatly in reducing the tissue sample to a fine state of subdivision, even to the more intimate disintegration of the cells. Furthermore, Meyerhof and Lohmann have pointed out (12) that freezing of the tissue actually accelerates the hydrolysis of phosphoarginine. Since phosphoarginine is not hydrolyzed by arginase it is essential, of course, that the phosphoarginine be fully converted into arginine and the promotion of its hydrolysis in this manner is clearly helpful.

Ordinarily 5 gm. samples of the frozen, powdered, muscle were weighed out and treated with 5 cc. of 10 per cent sodium tungstate, 5 cc. of 0.66 N sulfuric acid, and 45 cc. of water. If the arginine content was suspected to be high, 2 gm. samples treated

with 2 cc. volumes of the precipitating reagents and 54 cc. of water were used. In other cases where little material was available for analysis and the arginine content was suspected to be low, samples of only 1 or 2 gm. were used, with 1 or 2 cc. portions of the precipitants and only 20 or 30 cc. of water.

As originally described, the tissue suspension was shaken for several minutes and then filtered. For the purposes of

TABLE I  
*Determination of Optimum Conditions for Hydrolysis of Phosphoarginine in Muscle*

Genus and species	Arginine per 100 gm. muscle treated in acid suspension			
	0 to 1 hr. at 22°	8 to 10 hrs. at 22°	21 to 25 hrs. at 22°	2 min. at 100°
	mg.	mg.	mg.	mg.
<i>Haliotis cracherodii</i> .....	564	622	622	
“ <i>rufescens</i> .....	504	620	635	712
<i>Schizothærus nuttallii</i> .....		420	456	484
<i>Haliotis cracherodii</i> .....		616	634	663
<i>Cancer antennarius</i> .....			800	855
<i>Pachygrapsus crassipes</i> .....			717	771
<i>Orchestoidea californiana</i> .....			537	618
<i>Cancer antennarius</i> .....			809	804
<i>Paroctopus apollyon</i> .....			93	83
<i>Balanus nubilis</i> .....			669	658
<i>Leptoplana acticola</i> .....			18	33
<i>Pugettia producta</i> .....			760	825
<i>Paroctopus apollyon</i> .....			180	177
<i>Urechis caupo</i> .....		40	78	0
“ “.....			0	0
<i>Eudistylia polymorpha</i> .....			198	0
<i>Lumbricus</i> .....			100	16
“.....			94	18
<i>Dendrostoma zostericola</i> .....			64	Trace

investigation a different procedure had to be followed in order to insure complete hydrolysis of the phosphoarginine. As a rule, either of two alternative procedures was found advisable. To the weighed sample were added the requisite amount of water and one-half of the sulfuric acid. The tissue suspension, so obtained, was well shaken and then either heated to boiling in a water bath

and cooled after 2 minutes or it was permitted to stand at room temperature for approximately 24 hours. To the sample treated in either of these fashions, were then added the solution of sodium tungstate and the remaining half of the 0.66 N sulfuric acid. After a few minutes of standing the samples were filtered. Table I shows the effect of various treatments on the apparent arginine content of muscle. It should be mentioned that in the analysis of vertebrate muscle, where the arginine content is very low, the samples were prepared according to the original procedure.

Addition to the filtrates of the carbonate-bicarbonate buffer and arginase (Hunter and Dauphinee (13)) proceeded as described in the original method, except that the controls received 3 cc. of water in place of the arginase added to the experimental samples.

*Clarification of Samples*—After standing at 25° for 18 to 21 hours, 5 cc. of 1.5 M barium acetate were added to each. The samples were well shaken and filtered.

*Precipitation of Dixanthydryl Urea*—A 5 cc. portion of the clarified sample was placed in a 15 cc. centrifuge tube and mixed with 5 cc. of glacial acetic acid. 0.5 cc. of the solution of xanthydrol in methyl alcohol was added.<sup>2</sup>

*Oxidation of Dixanthydryl Urea*—The precipitated samples, after being washed and dried, were treated with potassium dichromate and sulfuric acid as described. In titration, we used larger quantities of water (200 cc.) than described by Kiech, Luck, and Smith and 20 cc. of potassium iodide instead of the 10 cc. as originally recommended.<sup>3</sup>

### *Experimental Animals*

All but one of the invertebrates used in this investigation were marine. They were collected from Monterey Bay and kept in aquaria until killed for analysis. Most of the animals were used within a day or two of capture.

<sup>2</sup> We have found it best to synthesize the xanthydrol ourselves (14) instead of depending on the commercial product. Owing to the instability of xanthydrol, especially in the solid state, the commercial preparations, unless quite fresh, have suffered partial conversion into dixanthyl ether and are of low solubility.

<sup>3</sup> We are indebted to Mr. Alton Kurtz for this suggestion. For reasons which will be described elsewhere the use of a larger quantity of potassium iodide enhances, appreciably, the accuracy of the titration.

In general, only muscle was used for analysis. Occasionally other organs were examined to learn whether the presence of arginine in appreciable quantity was limited to muscle. In the case of animals from which it was not feasible to dissect muscle, the whole organism was used.

### Results

Table II presents the results obtained on several common vertebrates. Of the species studied, only the rat and rabbit

TABLE II  
*Analysis of Vertebrate Muscle*

Animal	No. of specimens	Nutritional state	Urea per 100 gm.	Arginine per 100 gm.*	Amino N per 100 gm.
			mg.	mg.	mg.
Rat, male.....	4	Fasted 36 hrs.	3.9-26.9	3.39; 4.81; 7.86; 7.8	55.5-59.1
“ female....	2	“ 36 “	4.1; 10.1	5.68; 12.15	54.3
“ “ ....	2	Not fasted	18.9; 23.6	5.7; 0	
Rabbit, male...	2	Fasted 48 hrs.	57.6; 63.3	7.14; 4.15	47.7; 51.6
“ female	3	Not fasted	50.1-77.3	3.02; 6.55; 0	47.5
“ male...	1	“ “	22.9	9.66	
Pigeon, female	2	Fasted 48 hrs.	10.3; 17.7	0; 0	
Cat, male.....	2	“ 48 “	19.7; 46.3	0; 0	55.8; 71.9
“ female....	2	“ 48 “	42.1; 45.9	0; 0	
Frog, male....	3		5.6-7.1	0; 0; 0	16.4-18.2
“ female....	3		4.0-10.5	0; 4.3; 0	17.6-21.7
Gopher snake, male.....	1		2.2	0	79.4
Gopher snake, female.....	4		2.1-2.9	0; 4.3; 0; 0	92.1-95.9

\* Each value reported in this column is the average of duplicate determinations made upon a single specimen.

contained arginine in concentrations that permitted estimation. None could be detected in the pigeon, cat, frog, and gopher snake. Even in the rat and rabbit the quantities were very small compared to those found in invertebrates (Table III); so small indeed that to permit their exact determination a sensitive direct method would apparently have to be employed. In the estimation of arginine by an indirect method such as ours, it is clear that the

presence of much urea detracts from the precision of the values. This difficulty did not arise in the analysis of marine invertebrates in which the urea content is of negligible magnitude.

It will be observed that the values now reported for the arginine content of rat muscle are appreciably lower than those reported by Kiehn, Luck, and Smith. The difference, we find, is entirely due to the improved method of clarification by which contaminating impurities are completely removed.

TABLE III  
*Arginine Content of Invertebrate Muscle*

Genus and species	No. of determinations	Arginine per 100 gm.	Portion used
		mg.	
<b>Cœlenterata</b>			
<i>Aglaophenia struthionides</i> .....	1	0	Whole organism
<i>Evactis xanthogrammica</i> .....	1	0	Contractile portions of body wall
<i>Metridium dianthus</i> ....	2	0, 11	“ “
<b>Platyhelminthes</b>			
<i>Leptoplana acticola</i> ...	2	23 5*, 33	Whole animal. About 50 specimens per determination
<b>Phoronidea</b>			
<i>Phoronopsis harmeri</i> ...	2	10*, 14.6	Whole animal. About 25 specimens per determination
<b>Echinodermata</b>			
<i>Pisaster ochraceus</i> .....	2	161, 110	Tube-feet
<i>Patiria miniata</i> .....	1	60.5	“
<i>Pycnopodia helianthoides</i> .....	1	178	“
<i>Stichopus californicus</i>	2	0 211, 253 30	Wall Longitudinal muscle Circular muscle
<i>Strongylocentrotus franciscanus</i> .....	2	21, 38	Jaw muscle, 3 or 4 specimens per determination

TABLE III—Continued

Genus and species	No. of determinations	Arginine per 100 gm.	Portion used
		mg.	
<b>Annelida</b>			
<i>Eudistylia polymorpha</i>	2	0, 0	Lateral anterior muscle, 4 specimens per determination
<i>Glycera</i> .....	1	0	Muscle of body wall
<i>Polynoe brevisitosa</i> ....	1	0	Whole animal (2 specimens)
<i>Nereis vaxillosa</i> }.....	1	0	Whole animal (8 specimens)
<i>Lumbricereis</i> }			
<i>Lumbricus</i> .....	2	16, 18	Whole animal (15 specimens)
“.....	1	7	Muscle of body wall
<b>Sipunculoidea</b>			
<i>Physcosoma agassizii</i> ...	1	0	Muscle of body wall, 6 specimens
<i>Dendrostoma zostericola</i> .....	1	Trace	Muscle of body wall, 10 specimens
		“	Retractor muscles, 10 specimens
<b>Echiuroidea</b>			
<i>Urechis caupo</i> .....	4	0, 0, 0, 0	Muscle of body wall
<b>Arthropoda</b>			
<i>Crago stylirostris</i> .....	3	581*, 718, 678	Abdominal muscle, 3 to 5 specimens per determination
<i>Callinassa californiensis</i> .....	1	559	“ “
<i>Pachygrapsus crassipes</i> .....	2	547*, 771	Leg muscles (pincers)
<i>Cancer antennarius</i> ....	4	619*, 855, 804, 685	“ “ “
<i>Pugettia producta</i> .....	2	241*, 825	“ “ “
<i>Scyra acutifrons</i> .....	1	247	Abdominal muscle
<i>Loxorhynchus crispatus</i>	1	623	“ “
<i>Orchestoidea californiana</i> .....	1	618	Legs and abdominal muscle
<i>Balanus nubilis</i> .....	2	509*, 658	Adductor muscle

TABLE III—*Concluded*

Genus and species	No. of determinations	Arginine per 100 gm.	Portion used
		mg.	
<b>Mollusca</b>			
<i>Mytilus californianus</i> ..	2	244*, 183*	Adductor muscle
<i>Schizothærus nuttallii</i>	3	173*, 295, 173	" "
		278*, 484, 367	Foot and siphon muscle
<i>Paphia staminea</i> .....	2	98, 217	Adductor muscle
		186, 460	Foot and siphon muscle
<i>Cryptochiton stelleri</i> ...	1	120	" muscle
<i>Haliotis cracherodii</i> ...	4	338*, 622, 663, 441	" "
<i>Haliotis rufescens</i> .....	1	712	" "
<i>Tegula funebris</i> .....	1	194	" " 4 specimens
<i>Polinices lewisii</i> .....	1	332, 177†	" "
<i>Argobuccinum oregonensis</i> .....	2	195, 283	" "
<i>Doriopsis fulva</i> .....	2	0*, 0	Feet, 2 or 3 specimens per determination
<i>Anisodoris nobilis</i> .....	2	0, 8	Feet, 2 specimens per determination
<i>Hermisenda crassicornis</i> .....	1	19	Foot muscle
		491	Jaw muscle
<i>Paroctopus apollyon</i> ....	2	83†, 177	Arm "
		478§	Mantle muscle
		312	Funnel "
<b>Protochordata</b>			
<i>Clavellina</i> .....	1	0	About 25 organisms used whole

\* Immediately after weighing the sample, water, sodium tungstate, and sulfuric acid (full amount) were added. The suspension was filtered within 1 to 2 hours, and the protein-free extract measured out and treated with arginase 1 to 5 hours later. Phosphoarginine hydrolysis is therefore incomplete and the final value for total arginine is low.

† Same specimen, 2 to 4 days later.

‡ The same specimen 3 days later in a flaccid and dying condition gave a value of 19.

§ This sample, inadvertently, was boiled for 10 minutes instead of 2 minutes.

In Table III the results of 72 determinations made upon forty-one different species of marine invertebrates and one terrestrial form are presented. For purposes of discussion it will be convenient to consider the findings phylum by phylum.

*Cœlenterata*—Arginine is probably absent from the Hydrozoa and Actinozoa. Our observations in this respect confirm the work of Needham, Needham, Baldwin, and Yudkin (10) who showed that phosphagen is lacking from the Actinozoa. Ackermann, Holtz, and Reinwein (15) have shown that *Geodia* contains no arginine. We had hoped to obtain representatives of the Scyphozoa and Ctenophora but our efforts to do so were unsuccessful. Arginine determinations upon the latter would be of interest in view of the fact that they are reported to contain a low concentration of phosphagen (10).

*Platyhelminthes and Phoronidea*—The muscle arginine of the species examined may be greater than the values in Table III suggest. Owing to the small size of these animals we were obliged to use the entire organism in parts of which arginine would probably occur in much lower concentration than in muscle.

*Echinodermata*—The tube-feet of starfish and the jaw muscles of sea urchins contain arginine. It is worthy of note that the circular muscles lying within and adjacent to the body wall of the sea cucumber contain much less arginine than the longitudinal muscles. The isolation of arginine from *Arbacia pustulosa* was previously reported by Holtz and Thielmann (16) and from *Holothuria tubulosa* by Kutscher and Ackermann (6).

*Annelida*—To our surprise, arginine could not be found in the marine representatives of this phylum. The fact that Kutscher and Ackermann, despite earlier unsuccessful attempts (17), have now demonstrated the presence of arginine in *Lumbricus* (6) led us to investigate this genus. Several observations of interest arose in the course of the work. First of all we were able to confirm the recent findings of Kutscher and Ackermann. The quantities present in the animal as a whole were very small but were essentially the same whether or not liquid air was used in preparing the sample for analysis. In eviscerated specimens, despite the larger proportion of muscle a still smaller value was obtained. This suggests to us the possibility that most of the arginine estimated to be in the whole animal is present in the

viscous or as digestive products in the alimentary canal. It raises in our minds a doubt as to whether *Lumbricus* phosphagen (10) can possibly be phosphoarginine.

These doubts were heightened somewhat by a curious property of *Lumbricus* in acid suspension. In two of the determinations reported in Table III duplicate portions of tissue were weighed out and after acidification were permitted to stand for 21 to 25 hours at room temperature instead of being heated to 100° for 2 minutes. Both methods are known to be equally satisfactory for the hydrolysis of phosphoarginine. The observations recorded in Table I indicate, in fact, that the heating method applied to echinoderm, arthropod, and mollusc muscle is even slightly more effective. Nevertheless, there were four exceptions to the apparent rule that the heating method yields the larger values, and one of these exceptions is to be found in *Lumbricus*. Of the two duplicate samples mentioned above, in which the acidified tissue suspension remained at room temperature for 21 to 25 hours, one showed an arginine content of 100, and the other 94 mg. per 100 gm. of tissue. The remaining three exceptions were the annelid, *Eudistylia polymorpha*, the sipunculid, *Dendrostoma zostericola*, and the echinuroid, *Urechis caupo*, for which values of some magnitude were obtained when the suspension stood at room temperature (cf. Table I).

In our opinion this unusual behavior of *Lumbricus*, *Eudistylia*, *Dendrostoma*, and *Urechis* does not indicate the presence of phosphoarginine. It suggests instead the occurrence of a different arginine precursor, which by enzymic activity in an acid medium liberates arginine. As far as our studies have gone, only annelid, sipunculid, and urechis muscle behave in this way. It should be pointed out that rat muscle, which contains but little preformed arginine, does not yield larger quantities when permitted to stand in acid suspension. Rat muscle is therefore free of the new arginine precursor. Since the polychæte worms studied by Needham, Needham, Baldwin, and Yudkin (10) contain a relatively stable phosphagen, it is not unlikely that a new phosphagen, characteristic of the annelids and closely related phyla, may exist.

*Sipunculoidea*—Our failure to find arginine in this phylum disagrees with the observations of Meyerhof (9) who not only demonstrated the presence of a phosphagen resembling crustacean

phosphoarginine in its rate of hydrolysis, but also showed by the arginase method that arginine was present, unquestionably, in extracts permitted to stand for 15 hours at 25°. Perhaps this discordance is more apparent than real for it is not inconceivable that *Sipunculus nudus*, the species studied by Meyerhof, differs fundamentally in respect to its arginine content, from the small sipunculids, *Physcosoma agassizii* and *Dendrostoma zostericola*, examined by us.

*Arthropoda*—Relatively large quantities of arginine were found throughout the crustacea. We plan to investigate representatives of other classes later.

*Mollusca*—Next to the Arthropoda the largest concentrations of arginine were found in the members of this phylum. Nevertheless the distribution is far from uniform. Thus the nudibranch foot (*Doriopsis*, *Anisodoris*, *Hermisenda*) contains not more than a trace, if any, of arginine. The jaw muscles, however, which are surprisingly large in *Hermisenda*, are quite rich in arginine. Furthermore, the adductor muscles of clams have a much lower content of this substance than the muscles of the foot and siphon.

Among the cephalopods, *Paroctopus apollyon* was the only species available for study. At a later date we hope to investigate this order more exhaustively for the literature reveals several discrepancies which can only be clarified by further research. Morizawa (18) examined 50 kilos of muscle from *Octopus octopodia*. Guanine, adenine, hypoxanthine, xanthine, histidine, guanidine, cytosine, carnitine, taurine, betaine, and a base to which he gave the name octopin were isolated. Creatine or creatinine was alleged to be present. Arginine could not be found. Morizawa attached much importance to the betaine, which was present in considerable quantities and which Hoppe-Seyler and Linneweh (19) have shown to be of special metabolic significance as the principal nitrogenous constituent of cephalopod urine. Iseki (20) also demonstrated the presence of much betaine in octopus muscle in addition to two nitrogenous bases, one of which resembled methyl agmatine. Apparently arginine was not isolated. Meyerhof (9) states that the mantle muscle of cephalopods is free of phosphagen—a finding which Needham, Needham, Baldwin, and Yudkin (10) were unable to confirm. The concentrations of phosphoarginine found by the latter were quite as large as those

reported for other species listed in the table published by Needham and Needham (11). Arginine is also present in *Sepia* (21). Of the two specimens which we examined<sup>4</sup> the one giving the lower value for arginine in the tentacle muscle was quite young and weighed only 150 gm. The other specimen was a full grown adult of 25 kilos and contained a much higher percentage of arginine. This suggests that the arginine content of invertebrate muscle may show the same dependence on age as creatine in vertebrate muscle for it is well known that the muscle of the young animal has a lower concentration of creatine than that of the adult (22). As

TABLE IV  
Arginine Content of Other Organs

Species	Organ	Arginine per 100 gm muscle
		mg.
<i>Pugettia producta</i> .....	Muscle	241
" ".....	Ova	65
<i>Schizothærus nuttallii</i> .....	Adductor muscle	173
" ".....	Crystalline style	0
<i>Haliotis rufescens</i> .....	Muscle	620
" ".....	Contents of digestive gland	30 4
<i>Pisaster ochraceus</i> .....	Tube-feet	161
" ".....	Ripe ova	127
<i>Orchestoidea californiana</i> .....	Muscle	618
" ".....	Ripe ova	190
<i>Loxorhynchus crispatus</i> .....	Muscle	623
" ".....	Ripe ova	108

Table III also indicates, the arginine content of the mantle and funnel muscles is even greater than in the tentacles.

In Table IV we have collected the results of several estimations of arginine in organs other than muscle. It shows that arginine is contained in moderate concentrations in ova.

#### SUMMARY

1. The distribution of non-protein arginine (free arginine + phosphoarginine) in vertebrates and invertebrates has been studied.

<sup>4</sup> Both animals were uninjured in capture and were used for analysis while in excellent condition, within a few hours after removal to aquaria.

2. In the thigh muscles of the rat and rabbit the concentration of arginine is very low. None could be found in the muscles of the pigeon, cat, frog, and gopher snake.

3. In the invertebrates studied, the highest concentrations were found in crustacean muscle. It is abundant in the foot, adductor, and siphon muscles of the pelecypods and gastropods, with the exception of the nudibranchs, from the foot musculature of which it is absent. Much arginine is present in the cephalopod, *Paroctopus apollyon*. In the Echinodermata, Platyhelminthes, and Phoronidea also, the presence of arginine has been demonstrated and its concentration determined.

4. It is shown to be absent from the muscles of coelenterates, marine annelids, echiuroids, and sipunculids.

We wish to express our most cordial thanks to the staff of the Hopkins Marine Station for the facilities and aid extended to one of us (J. M. L.) in the prosecution of a portion of this work. Dr. Denis Fox of the Scripps Institution for Oceanography kindly supplied us with the specimens of *Dendrostoma*.

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## THE DIGITALIS GLUCOSIDES

### VL. THE OXIDATION OF ANHYDRODIHYDRODIGITOXIGENIN. THE PROBLEM OF GITOXIGENIN

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(Received for publication, November 30, 1932)

Gitoxigenin has been definitely shown in earlier work to be a hydroxydigitoxigenin.<sup>1</sup> The general position of this extra hydroxyl group, which may be oxidized to carbonyl, was suggested by certain reactions of gitoxigenin which involved this group. For example, it was shown that the isomerization of the aglucone with alkali to isogitoxigenin did not involve the tertiary hydroxyl group ( $\text{OH}^1$ ) which normally participates in the formation of the usual isogenins, such as isodigitoxigenin and isostrophanthidin, and during which the double bond of the original  $\Delta^{6,7}$ -unsaturated lactone group of the aglucone disappears with the formation of an oxidic ring as shown in Formulas I and II. Instead, the oxidic ring forms on the extra hydroxyl group which is in question.<sup>2</sup> The ready production of this oxidic ring, as well as its persistence as a lactone ring in the oxidation product, isogitoxigenic acid, seemed to restrict this hydroxyl group to a position  $\gamma$  or  $\delta$  to carbon atom (11). Since the correlation of the digitalis aglucones with strophanthidin has made the present knowledge of the structure of the latter applicable to the former, only two positions would appear to come into consideration for the hydroxyl group in question; namely, carbon atom (1) and carbon atom (18). The latter appeared more likely and was therefore adopted provisionally to explain the relationship between digitoxigenin and gitoxigenin as given in Formulas III and IV.

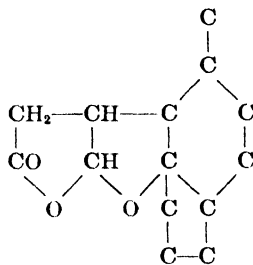
However, among the series of substances which were recently encountered in the determination of the position and size of Ring

<sup>1</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 199 (1930).

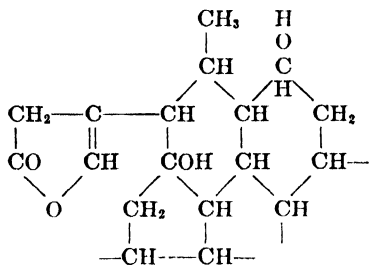
<sup>2</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **88**, 531 (1930).

The diagram shows the chemical structure of D-glucosamine, a six-membered ring with six chlorine atoms and six hydroxyl groups. The carbon atoms are numbered 1 through 18. The numbering is as follows: C1 is the top carbon, C2 is the carbon to its right, C3 is the carbon to the right of C2, C4 is the carbon to the right of C3, C5 is the carbon to the right of C4, and C6 is the carbon to the right of C5. The ring is completed by C7, C8, C9, C10, C11, and C12. The chlorine atoms are attached to C1, C2, C3, C4, C5, and C6. The hydroxyl groups are attached to C1, C2, C3, C4, C5, and C6. The numbering continues with C13, C14, C15, C16, C17, and C18, which are the carbons of the carboxylic acid groups attached to C1, C2, C3, C4, C5, and C6 respectively.

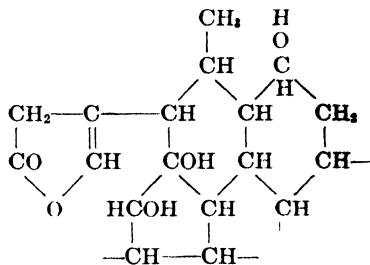
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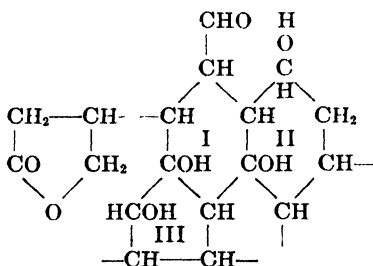
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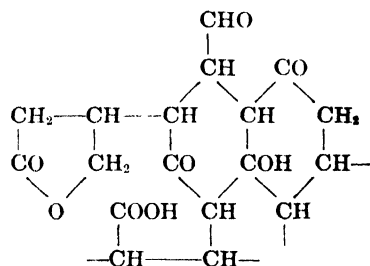
### III



IV

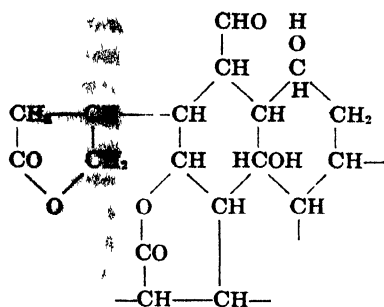


V

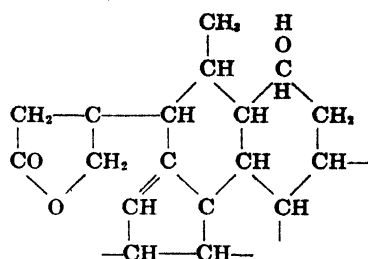


VI

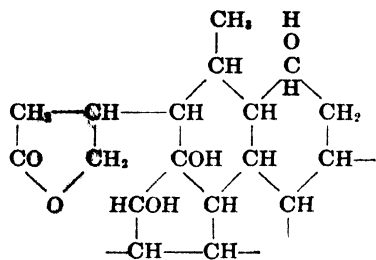
<sup>a</sup> Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **97**, 727 (1932).



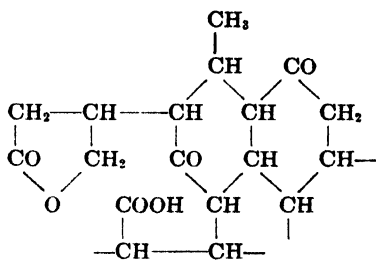
VII



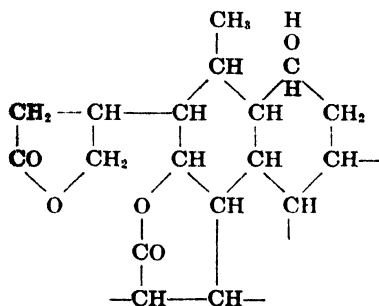
VIII



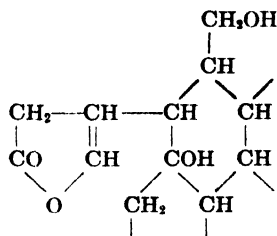
IX



X



XI



XII

The hydroxyl groups of hydroxydihydrostrophanthidin (Formula V) are thus in the same position as assumed above in the case of gitoxigenin (Formula IV), and it should be expected that the behavior of dihydrogitoxigenin on oxidation with chromic acid would parallel exactly that of the former. The course of the

oxidation, however, has been shown in a previous communication<sup>2</sup> to be quite different. Two substances were formed from dihydrogitoxigenin. A neutral dioxo derivative preponderated and in smaller amount a still unexplained acid was obtained apparently of the formula  $C_{23}H_{32}O_6$ . This discrepancy at once brings in question either the correctness of the interpretation of the series of reactions involved in the transformations from monoanhydrodihydrostrophanthidin or of the position given to the extra hydroxyl group of gitoxigenin. A means to check this point was found in a study of an analogous series of substances starting with dihydrodigitoxigenin.

Dihydrodigitoxigenin, as already described by Windaus and Stein,<sup>4</sup> is readily converted into anhydrodihydrodigitoxigenin (Formula VIII). The latter, after saponification, we have found can be readily oxidized with permanganate to form a hydroxydihydrodigitoxigenin (Formula IX). This substance was definitely different from dihydrogitoxigenin. In its further transformations it paralleled exactly the experience with monoanhydrodihydrostrophanthidin. On oxidation with chromic acid a diketolactone acid,  $C_{23}H_{32}O_6$  (Formula X), was formed, one of the carbonyl groups naturally arising from the secondary hydroxyl (OH)<sup>III</sup> of the aglucone. This substance did not correspond to the apparently isomeric acid mentioned above, obtained on oxidation of dihydrogitoxigenin, since titration and saponification readily showed it to be a monobasic lactone acid, as in the case of the strophanthidin derivative (Formula VI). Like the latter, on catalytic hydrogenation one of the carbonyl groups was reduced to hydroxyl, on which the carboxyl group then closed with the formation of a new lactone group. Simultaneously the second carbonyl group was also reduced to hydroxyl so that the resulting substance is a neutral hydroxydilactone,  $C_{23}H_{34}O_5$  (Formula XI).

The parallelism of these transformations with the experience with monoanhydrodihydrostrophanthidin substantiates the interpretation given to the series of reactions previously reported with the latter substance. Gitoxigenin therefore cannot have the formula previously adopted as in Formula IV. Placing the extra hydroxyl group as a primary hydroxyl on carbon atom (1),

<sup>4</sup> Windaus, A., and Stein, G., *Ber. chem. Ges.*, **61**, 2438 (1928). Stein, G., Inaugural dissertation, University of Göttingen, 72 (1929).

as given in Formula XII, is also not in harmony with certain observations which have been made with dihydrostrophanthidol,<sup>5</sup> a substance which possesses such a hydroxyl group on carbon atom (1). On dehydration of the latter with acid, a monoanhydro derivative, *monoanhydrodihydrostrophanthidol*, was obtained, whereas dihydrogitoxigenin gives a dianhydro derivative like gitoxigenin itself. The position of the extra hydroxyl group of gitoxigenin is therefore a point which still remains to be determined. We are at present continuing efforts in this direction.

#### EXPERIMENTAL

*Anhydrodihydrodigitoxigenin*.—Anhydrodihydrodigitoxigenin which was used for the following oxidation was prepared from dihydrodigitoxigenin, as described by Windaus and Stein.<sup>4</sup> On recrystallization from a small volume of alcohol it formed platelets which melted at 182–185°.

*Hydroxydihydrodigitoxigenin*.—A solution of 0.6 gm. of the above anhydro derivative in 6 cc. of pyridine was treated with 7.2 cc. of 0.5 N sodium hydroxide solution. The initial precipitate rapidly dissolved as saponification of the lactone group occurred. The solution was at once diluted with 30 cc. of water and then treated in portions with 5 cc. of 5 per cent potassium permanganate solution. Toward the end, the manganate color persisted. The filtrate from MnO<sub>2</sub> was just neutralized to phenolphthalein with hydrochloric acid and was then concentrated on the water bath to remove the pyridine. During this process copious crystallization occurred. The substance, which proved to be neutral, was collected with water. On acidification of the filtrate to Congo red, an additional amorphous precipitate was formed which could be crystallized from dilute acetone and consisted essentially of the same substance as the first crystalline fraction.

The substance crystallized from dilute acetone as needles which melted at 193–196°.

4 350 mg. substance:	3 595 mg. H <sub>2</sub> O,	11 272 mg. CO <sub>2</sub>
4 346 " "	3 615 " "	11 193 " "
	C <sub>23</sub> H <sub>36</sub> O <sub>8</sub> . Calculated. C 70 36, H 9 25	
	Found. (a) " 70 67, " 9 25	
	(b) " 70 23, " 9 31	

<sup>5</sup> Jacobs, W. A., *J. Biol. Chem.*, **88**, 528 (1930).

*The Diketo Acid*,  $C_{23}H_{32}O_6$ —0.25 gm. of the above substance was dissolved in 6 cc. of 90 per cent acetic acid. The solution was treated with 2 cc. of Kiliani  $CrO_3$  solution. Oxidation was prompt. After 10 minutes the mixture was diluted, causing a slight turbidity. The further addition of saturated ammonium sulfate solution produced a pasty precipitate followed by crystallization on rubbing and standing. After 24 hours this was collected with water. The substance was recrystallized by concentration of its acetone solution and careful dilution to incipient turbidity. The acid separated slowly as stout, well formed prisms, which did not exhibit a sharp melting point. After sintering above  $192^\circ$  it slowly melted at  $196$ – $200^\circ$ .

The monobasic lactone character of the substance was shown by the fact that it consumed 1 mol of alkali on direct titration and an additional equivalent on saponification as follows: 12.472 mg. of substance in 2 cc. of alcohol were titrated directly with 0.1 N NaOH against phenolphthalein. Calculated for 1 equivalent, 0.309 cc.; found, 0.318 cc. After the addition of 3 cc. of 0.1 N alkali to the above mixture, it was refluxed for 2.5 hours and again titrated back. Found, 0.342 cc.

4 525 mg. substance: 3 225 mg  $H_2O$ , 11 365 mg.  $CO_2$

4 120 " " : 3 005 " " 10 323 " "

$C_{23}H_{32}O_6$  Calculated C 68 27, H 7 98

Found. (a) " 68 50, " 7 97

(b) " 68 33, " 8 16

*The Hydroxydilactone*,  $C_{23}H_{34}O_5$ —0.2 gm. of the previous diketo acid was hydrogenated in ethyl acetate solution with 0.1 gm. of platinum oxide catalyst. The sparingly soluble substance dissolved as the reaction proceeded. In 1 to 2 hours absorption practically ceased after about 2 mols of hydrogen had been absorbed. The filtrate from the catalyst was concentrated to dryness. The residue was dissolved in chloroform. The solution was then shaken out with dilute sodium carbonate solution in order to remove any acid material. The washed chloroform solution of neutral substance was concentrated and diluted with ether. Crystallization readily occurred. On recrystallization by concentration of its solution in alcohol to small volume, the dilactone crystallized as needles. On rapid heating, it suddenly melted at  $145^\circ$  but at once resolidified and then melted sharply at  $232$ –

234°. Whether this behavior was due to solvent of crystallization or dimorphism was not investigated.

Due to its two lactone groups, the neutral substance consumed 2 equivalents of alkali on saponification as follows: 5.912 mg. of substance were boiled for 4.5 hours in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH and the mixture was then titrated back against phenolphthalein. Calculated for 2 equivalents, 0.303 cc.; found, 0.294 cc.

For analysis the substance was dried in the microdryer at 110°.

4.142 mg. substance: 3.295 mg. H<sub>2</sub>O, 10.745 mg. CO<sub>2</sub>

4.135 " " : 3.340 " " 10.710 " "

C<sub>23</sub>H<sub>34</sub>O<sub>5</sub>. Calculated. C 70.72, H 8.78

Found. (a) " 70.75, " 8.90

(b) " 70.64, " 9.03

*Monooanhydrodihydrostrophanthidol*—0.3 gm. of dihydrostrophanthidol<sup>5</sup> was dissolved at 0° in 7 cc. of HCl (1.19) and kept in ice for 45 minutes. The mixture was then diluted with water and the reaction product was salted out with ammonium sulfate. After recrystallization from benzene, it melted at 175–176°.

4.077 mg. substance: 3.230 mg. H<sub>2</sub>O, 10.590 mg. CO<sub>2</sub>

C<sub>23</sub>H<sub>34</sub>O<sub>5</sub>. Calculated, C 70.72, H 8.78; found, C 70.84, H 8.87



## THE ~~USE~~ OF CYSTEINE CUPROUS MERCAPTIDE IN THE DETERMINATION OF CYSTINE\*

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(Received for publication, December 2, 1932)

The colorimetric methods of Folin and Marenzi (1), and of Sullivan (2), and the iodine titration method of Okuda (3) are widely used for the determination of the proportion of cystine yielded by proteins. Comparisons between the results secured by these methods have appeared in a number of investigations (4-6) and, in many cases, disagreements have been recorded.

In each of these procedures samples of protein hydrolysates that contain all of the products of the hydrolysis, save those removed by a decolorizing agent, are taken. There is always a possibility that some substance other than cystine may react in such a way as to vitiate the accuracy of the result, and also that some of the cystine may be absorbed by the decolorizing agent and lost.

It would seem that if, as a preliminary step, the cystine could be quantitatively precipitated from the hydrolysate of the protein, more trustworthy determinations of this amino acid would be secured. Many attempts to do this have been recorded but, in general, little success has been attained. Precipitation by phos-

\*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington. A preliminary report was presented at the meeting of The Federation of American Societies for Experimental Biology, at Philadelphia, April, 1932.

We wish to express our appreciation of advice and cooperation from Professor H. T. Clarke.

† Sterling Fellow in Yale University, 1931-32; Porter Fellow of the American Physiological Society, 1932-33.

photungstic acid, although nearly complete when pure *L*-cystine is taken, fails with the mixture of cystine isomers produced during acid protein hydrolysis (7, 8). Precipitation by the salts of heavy metals (9, 10) is precluded because of the oxidation of one-sixth of the cystine which takes place when silver (11), mercury, or copper (12, 13) salts are employed, and which may also occur with other metals.

The ease with which cystine can be quantitatively reduced to cysteine by means of tin and acid (14), and the great insolubility of the silver mercaptides of cysteine (11), suggested that it should be possible to precipitate the cystine derived from the hydrolysis of a protein as a cysteine mercaptide. Experiments with pure cystine showed that, after reduction with tin and sulfuric acid and removal of the tin as sulfide, more than 98 per cent of the nitrogen could be recovered in the precipitate if an excess of silver sulfate were added and the mixture were then chilled and neutralized to Congo red with sodium hydroxide. The necessity of removing the tin, which gives rise to reduction of the silver during the neutralization, caused us to search for a reagent that could be employed in the presence of the stannous ion. Cuprous oxide was found to have suitable properties for this purpose.

Cuprous oxide was first used as a reagent for thiol compounds by Hopkins (15), who employed it for the isolation of glutathione. Pirie (16) has recently described the cuprous mercaptide of cysteine, and our own preliminary experiments showed that when cuprous oxide is added to hydrolysates prepared by boiling proteins with sulfuric acid in the presence of tin, only small quantities of other nitrogenous substances occur as contaminations of the cysteine cuprous mercaptide. The freedom from humin of the hydrolysates, prepared in this way, eliminates the necessity for a decolorizing agent.

These observations furnish the basis of a method for the determination of cystine that should be of value not only for the analysis of proteins but also in other connections.

### *Method*

The quantity of protein taken should be selected so that at least 20 mg. of cystine will be obtained; approximately 2 gm. are usually

convenient. The protein is boiled, under a reflux condenser, with 10 times its weight of 8 N sulfuric acid for 24 hours, 2 to 3 gm. of cystine filings being added as soon as the greater part has dissolved. The hydrolysate is decanted from the undissolved tin, which is thoroughly washed, and the solution is diluted to about 5 times its original volume. A water suspension of cuprous oxide (Kahlbaum's product is satisfactory and convenient) is then added to the warm solution (40–45°), in small quantities (about 0.2 gm.) at a time, with efficient mechanical stirring. It is usually easy to follow the disappearance of the initial pink color due to the oxide, and the formation of the gray cuprous mercaptide; time is allowed between each addition for the oxide to disappear. The end of the reaction is shown by the permanent pink or dull-red color of the precipitate; if stirring is stopped unchanged oxide collects on the bottom of the beaker. As a rule less than 1 gm. of oxide will be required, although of course this varies with the quality of the oxide and the amount of cystine present. There is no danger involved in adding too much since the cuprous mercaptide of cysteine, unlike the analogous glutathione compound (15), is not dissolved by an excess of the reagent.

When precipitation is complete the solution is chilled to 10°, and 10 N sodium hydroxide is added drop by drop, with constant stirring, until the reaction is at pH 4 to 5 as determined by Congo red paper. As the neutralization proceeds the color of the supernatant fluid becomes faintly blue. It is essential to keep the solution cold and well stirred during the whole operation, and the addition of alkali should be stopped before the neutral point of Congo red has been passed; the solution is then chilled overnight.

The precipitate is centrifuged off and washed with water four or five times successively, or until it shows a tendency to pass into colloidal solution. The washing is best accomplished by shaking in the stoppered centrifuge bottle; the froth produced can be broken by the addition of a little alcohol from a wash bottle, and this also serves to prevent the precipitate from spreading in the film of water on the walls of the bottle. The precipitate is finally suspended in about 400 cc. of water, 1 to 2 cc. of concentrated

<sup>1</sup> As much as 10 gm. of a protein of unusually low cystine content, such as casein, should be taken.

hydrochloric acid are added, and the mercaptide is decomposed with hydrogen sulfide, preferably delivered under pressure with continuous mechanical shaking. The copper sulfide is centrifuged off, washed once with 200 cc. of water, and transferred to a beaker in which it is boiled for a few minutes with about 150 cc. of water made slightly acid with hydrochloric acid. It is then centrifuged and the extraction of the precipitate with boiling acidulated water is repeated three times more, or until a nitroprusside reaction can no longer be obtained. The necessity for this thorough washing was shown by the low results that were invariably obtained when it was omitted.

The solution and washings are concentrated *in vacuo* to about 150 cc. and the sulfate ion is removed from the cooled solution by the careful addition of a very small excess of cold saturated barium hydroxide. Usually not more than 5 cc. are required, but the presence of excess barium ion must be demonstrated by centrifuging a small sample and testing with very dilute sulfuric acid. The precipitate of barium sulfate is centrifuged off and washed four times in the same manner as the copper sulfide, suitably smaller quantities of acidulated water being employed. The solution and washings are combined, concentrated *in vacuo*, filtered, and evaporated with Denis' reagent for the determination of organic sulfur (17). It should be noted that a little barium is already present, consequently the solution obtained after the ignition with the Denis reagent must not be filtered before precipitation of the barium sulfate. The cystine content of the protein sample is calculated from the organic sulfur found.

It may occasionally be desirable to hydrolyze a somewhat larger sample of protein than that mentioned, and to determine both nitrogen and sulfur in aliquot parts of the final solution. This was done in most of the analyses reported in Table I. The proportions of cystine calculated from the nitrogen were in no case more than 10 per cent greater than those calculated from the organic sulfur and frequently checked closely; evidently, therefore, only a small proportion of nitrogenous substance other than cystine finds its way into the cystine solution.

*Cystine Yielded by Proteins*—In Table I are shown the results of determinations of the proportions of cystine yielded by a number of proteins. Four of the proteins listed have been analysed by

Folin and Marenzi, and, in fact, the sample of zein was from the same preparation they employed. The agreement between the figures indicates that the two methods lead to substantially similar results. In six cases we have analyzed the same proteins as Sullivan and again the agreement between the results is close. The data obtained by the Folin and Looney method (18) do not, in general, agree with ours, nor with those of Folin and Marenzi, but Folin and Marenzi have stated that their method is much more dependable than the earlier method of Folin and Looney. The Okuda method, in the hands of Sullivan and Hess (5), and of Teruuchi and Okabe (19), has also given results that agree fairly well with ours. It would seem, therefore, that, when the several procedures are properly conducted, the Folin-Marenzi, the Sullivan, the Okuda, and the cuprous mercaptide method are all capable of yielding trustworthy results, provided the test of reliability is taken to be agreement to within 10 per cent of the magnitudes measured; few amino acids can be determined with much higher precision than this.

Failure of agreement between different methods may, in some cases at least, be due to actual differences between the preparations of protein employed. An example of this possibility is shown in Table I in which results obtained upon three different preparations of casein are quoted. Casein A was a highly purified preparation made in this laboratory by the Hammarsten method, Casein B was a commercial casein of the highest quality,<sup>2</sup> Casein C was another commercial sample for which no especial claims for purity were advanced. The first two agree closely in respect to the yield of cystine, the last apparently contains traces of an impurity that yielded additional cystine. Another and more striking example is furnished by wool. Our specimen was a commercial product prepared for surgical dressings and was extracted with ether before analysis. The cystine value we have found lies within the range of 8.0 to 10.8 per cent observed by Wilson and Lewis (24), but is much lower than that reported by Sullivan and Hess (5), who found 12.8 per cent of cystine in their material by the Sullivan method, and 14.0 per cent by the Folin-Marenzi method. Specimens of English wool analyzed by Rimington (6) likewise yielded

\* We wish to express our thanks to Dr. I. F. Harris of the Harris Laboratories for this specimen.

TABLE I

*Cystine Yielded by Proteins*

The figures are percentages of the ash- and moisture-free substance.

Protein	Cuprous mercaptide method		Folin and Marenzi (1)	Sullivan and Hesse		Molnar, Loefer (19)	Molnar, Loefer method (20)	Barnstein, gasometric (21)
	Individual values	Averages		Colorimetric (5)	Okuda method (5)			
Edestin (hemp- seed)	1 29 1 21 1 18 1 20 1 27 1 31	1 25	1 35	1 22	1 32	0 75	0.97	1 79
Phaseolin (navy bean)	0 16	0 16		0 11 (22)*	0 16 (22)		0.58	
Globulin (tobacco seed)	0 90	0 90						
Gliadin (wheat)	1 97 2 15 2 06	2 06	2 18	2 16	2 44	2 32	2.42	2 74
Zein (maize)	0 92 0 90	0 91	1 03	0 85† 0 83		0 5	0.85	1 58
Casein A	0 20 0 21	0 21	0 30	0 30 (23)		0 25	0.26	0 66
“ B	0 22 0 24	0 23						
“ C (crude)	0 44 0 47	0 46						
Lactalbumin	2 55 2 56	2 56		2 29	2 44	3 98 (5)	3.77	
Fibrin	1 57 1 45	1 51			1 48 (19)	3 5	1.58 1.64	
Hemoglobin (horse)	0 43 0 39	0 41						
Wool	9 41 9 66	9 53						

reference numbers.

† We are deeply indebted to Professor Sullivan for determining the cystine yielded by our sample of zein.

from 12 to 13 per cent of cystine. The studies of Lightbody and Lewis (25) and of Smuts, Mitchell, and Hamilton (26) have shown that the proportion of cystine obtained from rat hair is markedly affected by the availability of cystine in the diet of the animal, and also by its age; the wide range of cystine determinations in different specimens of wool need not, therefore, be surprising.

Especially interest attaches to the determination of cystine in horse hemoglobin. So far as we are aware no convincing evidence of the presence of cystine in hydrolysates of this protein has hitherto been presented. The preparation we analyzed was made for us in Professor E. J. Cohn's laboratory at Harvard (27) and had been crystallized twice, coagulated, and extensively washed. A positive qualitative test for the presence of cystine in the final solution was obtained by Sullivan's method. The iron in the cystine fraction was removed as hydroxide before the determination of the organic sulfur was made. The necessity for this precaution was shown by the slightly higher values (0.50, 0.46 per cent) obtained when it was omitted, and by the obvious presence of iron in the barium sulfate. The proportion of cystine found agrees fairly well with the theoretical requirement (0.35 per cent) for 1 cystine molecule per molecule of hemoglobin of weight 66,400.

*Quantitative Precipitation of Cystine As Cysteine Cuprous Mercaptide*—A solution of 0.200 gm. of cystine in 40 cc. of 4 N sulfuric acid was boiled with tin for 1 hour, decanted, and diluted to 400 cc. The cysteine was precipitated as cuprous mercaptide as already described, was centrifuged off, washed once with water, and transferred to a Kjeldahl flask. In two experiments nitrogen equivalent to 0.199 and 0.195 gm. of cystine was found in the precipitates; in two other experiments, in which 0.100 gm. of cystine had been taken, nitrogen equivalent to 0.0979 and 0.0999 gm. of cystine was found: under these conditions, therefore, cysteine can be precipitated to the extent of at least 98 per cent.

*Recovery of Cystine Added to Hydrolysates of Casein*—In order to test the degree to which cystine can be recovered from hydrolysates of protein, experiments of three types were conducted. Casein (4 gm.) was hydrolyzed with 8 N sulfuric acid in the presence of tin, the hydrolysate was diluted with an equal volume of water, 0.1 or 0.2 gm. of cystine was added, and the solution was boiled for an hour. The procedure already described was then followed and

the sulfur found was calculated as cystine and corrected for the quantity of cystine originating from the casein; for this purpose the cystine content of the casein was taken as 0.2 per cent. In the second type of experiment 0.1 or 0.2 gm. of cystine was added to 5 gm. of casein, this was subjected to hydrolysis with 8 N sulfuric acid in the presence of tin, and the cystine found was corrected as before. In the third type of experiment a cystine-free hydrolysate was prepared from 50 gm. of casein by precipitation of the cysteine with cuprous oxide. The filtrate was treated with hydrogen sulfide to remove the tin and copper, was concentrated, and then treated with 2 volumes of alcohol to precipitate the sodium sulfate; this was dissolved in water and reprecipitated with alcohol, and the combined mother liquors were concentrated to a convenient volume. Aliquot parts that represented approximately 5 gm. of the original casein were taken, 0.1 or 0.2 gm. of cystine was added, together with sufficient 8 N sulfuric acid and tin, and the solution was boiled for an hour to reduce the cystine. The regular procedure was then followed.

Data from a number of experiments of each type are assembled in Table II. The average recovery of cystine added to casein before hydrolysis was 92.8 per cent, of that added after hydrolysis was 92.2 per cent, while the average recovery from the cystine-free hydrolysate was 94.6 per cent. It is evident that the partial racemization of cystine that occurs during hydrolysis of the protein did not interfere with the determination.

*Preparation of Cysteine Cuprous Mercaptide*—The precipitate that forms when an excess of cuprous oxide is stirred with a dilute sulfuric acid solution of cysteine is readily soluble in 8 N sulfuric acid; the excess of reagent, and any metallic copper, can then be removed by centrifugation. When the clear green solution is poured into a large volume of water, the cysteine mercaptide separates as a flocculent white precipitate.

Preparations were made in this manner from 2.0 gm. of cystine and were washed repeatedly by shaking with water in the centrifuge bottle; the first two or three wash fluids contained considerable sulfate, as might be expected, but an appreciable test for sulfate and for cupric copper could be obtained even after seven washings. At this stage the precipitate usually began to pass into colloidal solution; 50 per cent alcohol was therefore employed as

washings, and it was noted that the removal of sulfate and of cupric copper from the precipitate at once ceased. The last washings were conducted with absolute alcohol and then absolute ether. During the operations the product darkened to a dull olive-green color after being dried *in vacuo* over sulfuric acid to constant

TABLE II  
*Recovery of Cystine Added to Casein*

		Cystine				Cystine added
Casein taken	Added	Found	From casein	Recovered		
gm	gm	gm	gm	gm	per cent	
4 63	0 100	0 103	0 0092	0 094	93 9	After hydrolysis
4 74	0 200	0 193	0 0094	0 184	91 9	" "
4 65	0 200	0 191	0 0093	0 182	90 8	" "
Average					92 2	
5 280	0 100	0 101	0 0107	0 090	90 0	Before hydrolysis
5 232	0 100	0 100	0 0105	0 0895	89 5	" "
5 244	0 200	0 199	0 0104	0 189	94 3	" "
5 225	0 200	0 205	0 0104	0 195	97 4	" "
Average					92 8	
5 0	0 200	0 199			99 5	To cystine-free hydrolysate
5 0	0 200	0 186			93 1	" " "
5 0	0 200	0 180			89 9	" " "
5 0	0 200	0 193			96 5	" " "
5 0	0 200	0 174			86 8	" " "
5 0	0 100	0 098			98 4	" " "
5 0	0 100	0 098			98 0	" " "
Average					94 6	

weight, consisted of a somewhat hygroscopic powder with no evidence of crystalline structure. Analyses of preparations made in this manner did not give integral atomic relationships between the quantities of nitrogen, sulfur, copper, and water of hydration, but the figures suggested that the preparations contained an excess of copper together with sulfate sulfur equivalent to this extra

copper. The behavior of the precipitate during washing indicated that the compound, as freshly precipitated, contained cupric copper which was largely, but not entirely, removed by washing with water. Consequently preparations were made in which the washing, after a second precipitation in water, was conducted with absolute alcohol. Analyses of three of these are shown in Table III. The atomic ratios, calculated from the average of the results, are shown in the last line of Table III. It is obvious that the atomic ratio of the sulfate sulfur is equivalent to the excess over unity of the atomic ratio of the copper, and that there are 2 molecules of water for each sulfate sulfur atom; furthermore if the ratio of the sulfate sulfur is deducted from that of the total sul-

TABLE III  
*Cysteine Cuprous Mercaptide Precipitated in Water and Washed with Alcohol*

Preparation No	Nitrogen	Total sulfur	Sulfate sulfur	Copper	Water
	per cent	per cent	per cent	per cent	per cent
1	4.50	16.48	6.92	33.64	7.01
2	4.63	16.95	7.13	34.86	9.15
3	4.78	16.92	6.92	37.26	7.6
Average	4.63	16.78	6.99	35.25	7.92
Atomic ratios	1.00	1.58	0.66	1.67	1.28

fur, and also from the copper, the ratios of nitrogen-sulfur-copper in the balance of the molecule are very close to 1:1:1.

Further investigation will be required to ascertain the true nature of the precipitate that separates when the strongly acid solution of the mercaptide is poured into water. It contains cupric copper, and also sulfate, in equivalent proportions, but the exact chemical relationships of these to each other and to the rest of the compound are obscure. It is improbable from the method of preparation that they represent cupric sulfate admixed with the mercaptide.

From the purely empirical point of view our analytical findings are in accord with the assumption that the precipitate had a composition represented by the formula  $C_2H_4O_2NSCu \cdot CuSO_4 \cdot 2H_2O$  and that one-third of the cupric copper, the sulfate, and the water

of hydration was washed away in spite of the precautions that were taken.

That no material constituent part of the compound has been overlooked is evident from the following calculation: the cystine radical content, calculated from the organic sulfur, is 36.67 per cent; if to this are added the sulfate sulfur, the oxygen equivalent to the sulfate sulfur, the copper, and the water of hydration, the sum is 100.8 per cent.

During the development of the cuprous mercaptide method it was found that better results were obtained if the solutions were neutralized to Congo red after the addition of an excess of cuprous oxide. In order to see whether neutralization has any effect upon

TABLE IV  
*Cystine Cuprous Mercaptide Precipitated in Water, Neutralized, and Then Washed with Alcohol*

Preparation No	Nitrogen	Total sulfur	Sulfate sulfur	Copper	Sodium	Water
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1 20	20 90	18 19	13 12	24 50	1 08
2	1 24	20 26	17 42	12 70	23 96	0 28
Average	1 22	20 58	17 80	12 91	24 23	0 68
Atomic ratios	1 000	7 386	6 39	2 32	12 10	0 38

the mercaptide, preparations were made from 2 gm. of cystine in which the suspension of precipitate, after the addition of cuprous oxide, was slowly neutralized with sodium hydroxide. The precipitate was centrifuged off, dissolved in 200 cc. of 8 N sulfuric acid, centrifuged, and the clear green solution was poured into 2 liters of water. The mercaptide at once separated, and the supernatant fluid was then neutralized to Congo red as before. The precipitate was collected at the centrifuge and washed repeatedly with absolute alcohol and finally with ether. Analyses of two such preparations are shown in Table IV. The atomic ratios calculated from the average composition are shown in the last line. If the sodium be supposed to be present as sodium sulfate, precipitated from the adhering mother liquor by the alcohol used in washing the flocculent precipitate, it is clear that 12 sodium atoms and 6 sulfate

sulfur atoms may be set aside for sodium sulfate. The additional 0.39 atom of sulfate sulfur probably belongs with the extra 0.32 atom of copper, and there are left nitrogen, organic sulfur, and copper in the ratio 1:1:2. The fundamental compound in the precipitate obtained after neutralization appears, therefore, to be the cuprous salt of cysteine cuprous mercaptide,  $C_3H_5O_2NSCu$ .

*Recovery of Cystine from Mercaptide*—The cuprous mercaptide obtained from 2.00 gm. of cystine was washed with water and decomposed with hydrogen sulfide. After being freed from hydrogen sulfide by concentration *in vacuo*, the solution, together with the washings of the copper sulfide, was diluted to 1 liter and neutralized to pH 7.0 with barium hydroxide. The barium sulfate was removed and washed free from cysteine, and the combined solution was concentrated to 1800 cc. and aerated until a nitroprusside reaction could no longer be obtained. The solution was then turbid, but no definite separation of cystine had occurred; it was therefore concentrated *in vacuo* to 200 cc. and chilled. The cystine was collected quantitatively, weighed, dissolved in *N* hydrochloric acid and filtered from a trace of barium sulfate. The corrected weight of the cystine was 1.054 gm.; it contained 11.3 per cent of nitrogen (theory 11.66) and had a specific rotation of  $[\alpha]_D^{20} = -196^\circ$ . The crystals were exclusively hexagonal plates but the preparation was somewhat yellow in color.

The mother liquor was evaporated to 50 cc. and an equal volume of alcohol was added, whereupon a second crop of crystals separated. The mother liquor from this was evaporated to 20 cc., 2 volumes of alcohol were added, and the beaker was chilled several days. A small crop of impure cystine separated. The final mother liquor, when evaporated to dryness, left an insignificant residue. Corrected for the inorganic impurity the second and third crops weighed 0.56 and 0.22 gm. respectively. The total recovery therefore appeared to amount to somewhat more than 91 per cent of the cystine taken. The second and third crops were not, however, pure cystine. A part of the second crop remained insoluble on treatment with 20 cc. of boiling water and manifestly consisted essentially of cystine; its nitrogen content was equivalent to 0.193 gm. of this substance. The soluble part was analyzed colorimetrically and was found to contain 0.204 gm. of cystine; thus the

op consisted to the extent of about 80 per cent only of cystine. The third crop was even less pure.

It is clear, therefore, that the solution obtained after decomposing the cuprous mercaptide contains decomposition products of cystine. Among these are ammonia, traces of which were recognized by Nessler's test, and probably pyruvic acid, inasmuch as a faint iodoform reaction could be obtained and also a recognizable bichromate color with salicylaldehyde and alkali. These observations supply the explanation of our failure to obtain results that checked with the cystine, as calculated from the organic sulfur, when the Folin and Marenzi method was applied to our fractions after these had been aerated at pH 7 to convert the cysteine to cystine. Although numerous colorimetric determinations were carried out on these fractions, the results were invariably low.

#### DISCUSSION

The present method for the determination of cystine rests upon the assumption that the total quantity of thiol groups in the reduced hydrolysate arises from cystine originally present as cystinyl radicals in the protein. Whether or not this assumption is justified is uncertain; nevertheless no conclusive evidence of the presence in native proteins of preformed cysteine, or of other thiol or disulfide compounds, has hitherto been presented. The close agreement of our results with those obtained by Sullivan's highly specific color reaction supports the view that the determinations of organic sulfur in the cystine fraction obtained by the cuprous mercaptide method are in fact determinations of cystine. Experiments with methionine showed that this substance is not precipitated under the conditions we have employed.

The method, as at present developed, requires the use of sufficient protein to afford 20 mg. or more of cystine. There is, however, no apparent reason why the procedure could not be conducted on a smaller scale provided equipment for microanalysis were at hand. Waelsch and Klepetar (28) have recently described a slight modification of the Denis reagent which permits the accurate determination of the sulfur in 0.5 to 2.5 mg. of cystine. This may prove useful in the further development of the method.

## SUMMARY

A method is described for the determination of the cystine yielded by proteins, which depends upon hydrolysis of the protein by sulfuric acid in the presence of tin, and precipitation of the cysteine produced as cysteine cuprous mercaptide. The organic sulfur in the precipitate is determined and calculated as cystine.

The results of analyses of a number of proteins by this method agree closely with the results obtained by Folin and Marenzi, and by Sullivan, with their respective colorimetric methods, and by Sullivan with the Okuda iodine titration method. Support is thereby lent to the view that these methods, when properly conducted, yield trustworthy results. The present method possesses certain advantages in that no decolorizing agent is required, a separation of cystine from the products of hydrolysis is effected, and the somewhat intricate technique of the colorimetric methods is avoided.

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## DIET AND BLOOD CHOLESTEROL IN NORMAL WOMEN\*

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That the long continued feeding of diets rich or poor in fat has a definite effect upon the general level of blood lipid has been indicated by data on dogs recently published by Bloor (1). If we may assume that concentration of blood cholesterol in any way affects the deposition or accumulation of sterol in the tissues, we have reason to be concerned with the factors which affect the extreme variability of blood cholesterol level in human beings, and especially in women.

The purpose of the study here reported was 2-fold: (a) to gain some evidence of the effect in human beings of *continued* feeding of cholesterol-rich food upon postabsorptive values for blood cholesterol, and (b) to see if the monthly variations in blood cholesterol which we had previously observed (2) in normal women were affected by cholesterol intake.

Our plan called for an intensive study of a few subjects on diets of variable cholesterol content, but of quite a usual character. The first or control diet,<sup>1</sup> of constant and rather low fat as well as

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<sup>1</sup> *Detailed Description of Diets.* *Diet I*—This was a control diet which contained orange juice 230 gm., whole wheat bread 160 gm., butter 52 gm., jam 13 gm., lettuce 130 gm., lamb chops 92 gm., cream 26 gm., milk (1 pint) 467 gm., mayonnaise 13 gm., wheat germ 20 gm., potato 120 gm., two servings of vegetable of approximately 100 gm. each per day, and two of fresh fruit, usually apples. *Diet I*, the low cholesterol diet, contained, as far as we could ascertain from published data and from our own analyses, in cases where figures were not available, 0.77 gm. of cholesterol per day.

*Diet II*—This high cholesterol diet contained the yolks of four eggs (64 gm.) daily and on alternate days  $\frac{1}{2}$  pound (118 gm.) of liver was substituted for the lamb chops. The cholesterol content of this diet was calcu-

sterol content, was given for 4 or 5 weeks (one full monthly cycle). A few weeks respite from this routine were allowed and then the so called high cholesterol regimen (Diet II) was instituted. This consisted essentially of the same basal diet given before with egg yolk and liver added to bring the cholesterol content to 3.1 gm. daily and was continued for a similar period. Finally, after a break in the monotony of the regimen had again been allowed, the menstrual and intermenstrual response of these same subjects on the same basal diets to the ingestion of cholesterol as such was studied (Diet III). Unfortunately the latter period of observation had to be shortened because of the close of the school year.

Four normal women, all senior or graduate students in nutrition, served as subjects. Blood samples were taken before breakfast and at very nearly the same time in the morning three times a week; occasionally more frequent samples were taken at or near the time of onset of menstruation. The general technique described in previous papers of this series was followed.

Blood samples were measured immediately into alcohol-ether and filtrates prepared according to the technique of Bloor, Pelkan, and Allen (3). Total and free cholesterol were determined in the whole blood filtrates by precipitation and oxidation of the digitonide according to the method previously described by one of us (4). The modification of the procedure introduced by Yasuda (5) was adopted only in so far as the acetone washing of the digitonide was concerned. We should perhaps state that we have found it relatively easy to get dependable recovery of added cholesterol with asbestos filters when the concentrations in the samples lie within the ranges covered by normal bloods.

The determinations were made with whole blood—partly because we wished to avoid taking the larger samples necessary for plasma determinations; partly, however, because we are convinced that much error may be introduced into plasma cholesterol deter-

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lated as 3.1 gm. Amounts of bread and potato, vegetables, and fruit were omitted from this diet to make the caloric content approximately equal to that of Diet I without altering the fat and sterol content.

*Diet III*—This was a basal diet, essentially the same as Diet I, to which cholesterol was added (in the butter) to make the total content approximately equal to that of Diet II, i.e. 3 gm. This diet differed from Diet II in that the eggs and liver were omitted.

mination by variations in the distribution between plasma and corpuscles due to slight variations in concentration and consequently osmotic pressure introduced by the anticoagulant (possibly an indirect effect on emulsification) and by day to day variations in temperature and speed of centrifugation in the separation of the plasma. We wish, however, to reserve further comment on this phase of the subject until we have accumulated more data, and mention the point at this time only because of certain statements with regard to the use of plasma for cholesterol determinations in the recent literature. Detailed data are given in Table I, while mean results are summarized in Table II.

#### DISCUSSION

The question of relationship of diet to blood cholesterol is one on which the conflicting evidence in the literature can to some extent be accounted for by variations in conditions of experimentation, pathology of subjects used, and failure to appreciate limitations of the methods employed. In general the results of short time experiments are inconclusive. Bruger and Somach (6) have recently stressed the extent of diurnal variations in blood cholesterol, even in normal subjects. They conclude from data covering in each case observations taken at 2 hour intervals for from 4 to 24 hours that the ingestion of food has no appreciable effect, either early or late. Close examination of their figures shows, however, that all the cases studied after ingestion of food were pathological, and certain of their figures suggest the possibility of other interpretations, notably the effect of water ingestion or of progressive loss of water during fasting.

A number of papers dealing with the effect of single ingestions of cholesterol or cholesterol-rich foods present equally unsatisfactory data.

In a recent paper by Hunt (7) the literature on the subject is reviewed in connection with diabetes. Her conclusion from her own data is characteristic; *i.e.*, she states that egg-eating diabetics do not necessarily have high blood cholesterol values, but in the one case with high cholesterol from whose diet eggs were omitted, there was a real drop in the blood cholesterol level.

Myassnikow (8) got no measurable effect on blood cholesterol in normal subjects by a single feeding of eight eggs or in three

TABLE I  
*Cholesterol in Blood Following Diets of Varying Cholesterol Content*

Total and free cholesterol are measured in mg. per 100 cc. of blood; ester cholesterol is measured in both mg. per 100 cc. of blood and per cent of total.

Diet I, low cholesterol	Subject E-Tu				Subject M-Tu				Subject M-Hj				Subject G-Hj										
	Cholesterol			Date*	Cholesterol			Date*	Cholesterol			Date*	Cholesterol										
	Total	Free	Ester		Total	Free	Ester		Total	Free	Ester		Total	Free	Ester								
				per cent				per cent				per cent				per cent							
-3	190	88	103	54	7	-1	177	102	75	42	3	-4	149	105	43	28	8	+1	143	85	58	40	5
-1	167	89	78	46	6	0	173	98	75	43	4	-2	147	97	50	34	0	+3	149	83	66	40	4
+1	154	82	73	47	4	+1	152	89	63	41	4	0	151	100	51	33	7	+6	141	91	50	35	4
+2	149	92	56	37	6	+3	158	91	67	42	4	+2	151	100	51	33	7	+8	149	86	63	42	2
+4	156	109	47	30	1	+5	145	107	38	26	2	+5	145†	91†	54†	37	2	+10	147	73	73	49	6
+6	168	90	77	45	8	+8	149	94	55	36	9	+7	166†	94†	72†	43	3	-13	126	82	44	34	8
+9	152	102	50	32	8	+10	149	95	54	36	2	+9	151†	85†	66†	43	7	-12	138	77	61	44	1
+11	152	92	60	39	4	+13	137	82	56	40	8	+12	183†	95†	88†	48	0	-10	128	77	51	39	6
+13	160	104	56	38	7	-13	137	98	40	29	1	+14	151	82	69	45	6	-7	160	97	63	39	3
-12	201†	79†	122†	60	6	-11	149	84	65	43	6	-11	151	102	49	32	4	-6	168	90	77	45	8
-10	177†	91†	86†	48	5	-8	158	95	63	39	2	-8	156	103	53	33	9	-5	145	94	51	35	1
-8	179	104	75	41	8	-6	136	92	43	31	6	-6	173	106	67	38	7	-4	132	92	40	30	3
-5	154	92	62	40	2	-4	151	89	62	41	0	-4	190	113	77	40	5	-3	162	94	68	41	9
												-2	147	97	50	34	0	0	137	96	41	25	4
												-1	168	98	70	41	6	+1	154	106	49	31	1
												0	162	72	60	35	3						

Diet II, high cholesterol	-14	169	99	71	42.0	-12	158	87	71	44.3	-1	179	94	85	47.5	-1	139	88	52	37.4
	-12	184	101	84	45.6	-10	158	86	72	45.6	+1	169	96	73	43.2	+1	154	92	41	37.7
	-10	168	107	61	36.3	-8	162	88	74	45.5	+3	175	94	81	46.2	+4	188	95	93	49.4
	-7	183	110	73	39.3	-5	162	92	69	42.5	+6	186	113	73	39.2	+6	173	115	58	33.5
	-5	186	93	93	50.0	-3	154	93	61	39.6	+8	190	113	77	40.5	+8	162	82	90	49.1
	-3	168	91	76	45.2	-1	173	100	73	42.2	+10	188	110	77	41.4	+11	154	89	66	42.8
	0	166	89	77	46.3	+1	169	104	65	39.0	-12	184	110	74	40.2	-10	154	91	63	40.9
	+2	173	89	85	49.2	+2	184	88	96	52.2	-10	171	99	72	42.1	-8	154	105	49	31.8
	+4	182	112	70	38.4	+4	171	112	59	34.5	-8	168	112	56	33.3	-7	149	86	63	42.0
	+7	168	108	60	35.7	+6	184	98	87	46.2	-5	184	122	62	33.6	-5	149	93	56	36.2
	+9	158	108	50	31.6	+9	179	102	77	43.0	-3	179	111	68	38.5	-3	156	87	69	44.2
	-1	143	78	65	45.4	+11	168	91	76	45.2	-1	200	110	90	45.0	0	149	83	66	44.2
	+1	143	94	49	34.2	+13	177	100	77	43.5	0	180	97	83	46.1	+2	152	88	65	49.4
	+2	145	90	55	37.9	+16	173	115	58	33.5	+1	181	101	80	44.1	+4	139	90	49	35.2
	+3	156	88	69	44.2															
	+4	159	86	63	39.5															
Diet III, added cho- lesterol	-3	162	89	72	44.4							147	100	47	31.9	-2	135	88	47	34.8
	-2	152	89	64	42.0						-8	154	102	53	34.4	-1	147	90	57	38.7
	-1	158	89	69	43.0						-3	177	99	78	44.0	0	154	90	64	41.5
	0	143	90	53	37.1						-2	177	98	79	45.0	+1	160	100	60	37.5
	+2	151	93	57	37.7						-1	160	98	62	38.7	+3	171	107	64	37.4
	+10	169	83	86	50.8						0	159	91	68	42.7	+11	152	89	63	41.4
	+11	173	100	73	42.1						+1	164	94	70	42.6	+12	158	92	66	41.7
	+12	162	91	71	43.8						+2	168	97	71	42.2	+13	158	91	67	42.4
	+13	168	88	80	47.0						+3	166	97	69	40.9	+14	162	94	68	41.9
	+15	148	91	57	38.4															

\* The day of the onset of menstruation is recorded as 0; days preceding the onset of menstruation as (-); and days following it as (+).

† Samples taken when the subjects were suffering from slight colds.

TABLE II

## Mean Blood Cholesterol Values

The results are expressed in mg. per 100 cc. of blood.

Subject	Mean total cholesterol						Free cholesterol						Ester cholesterol					
	Diet I		Diet II		Diet III		Diet I		Diet II		Diet III		Diet I		Diet II		Diet III	
	P.e.		P.e.		P.e.		P.e.		P.e.		P.e.		P.e.		P.e.		P.e.	
E-Tu.....	162	±2.3	165	±1.6	159	±1.6	95	±1.7	96	±1.7	90	±0.9	67	±3.3	69	±2.1	68	±2.1
M-Tu.....	151	±2.3	169	±1.6			93	±1.3	97	±1.6			58	±2.5	73	±1.9		
M-Hj.....	158	±2.2	181	±1.7	164	±2.1	98	±2.1	106	±1.6	96	±0.7	62	±2.1	75	±1.6	66	±2.4
G-Hj.....	145	±2.2	155	±2.2	155	±2.2	88	±1.4	92	±1.9	93	±1.3	57	±1.9	64	±2.2	62	±1.4
Mean for diet.....	154.0		167.5		159.3		93.5		97.7		93.0		60.6		70.2		65.0	
Standard deviation..	7.5		8.5		5.1		4.9		4.3		0.2		3.5		0.3		3.3	
P.e. of mean.....	2.9		3.3		2.4		1.9		1.7		0.1		1.4		0.1		1.6	

Probable errors of means are computed by the formula p.e. of mean =  $0.6745 \sigma / \sqrt{n}$  where  $\sigma = \sqrt{\Sigma(d^2)/n - 1}$ . Figures representing values taken when the subjects were suffering from colds are omitted in computing means and probable error of means.

subjects by feeding eggs for a 4 day period. In one case of cirrhosis of the liver, however, he secured a tremendous rise in blood cholesterol level as a result of feeding eggs.

The differences in response to fat feeding, which were ascribed by Rony and Ching (9) to the previous state of nutrition of the experimental animal, together with the work of White and Hunt (10) who found that blood cholesterol values were distinctly higher in overnourished than in normal children, suggest one explanation of differences in response to diet in short time experiments. This effect of previous state of nutrition is also in keeping with the findings of Page and Menschick (11) who found (from balance experiments and tissue analyses in rabbits) that cholesterol tended to accumulate in the tissues of rabbits other than brain after feeding excessive amounts of cholesterol. They believe that their data establish ability of the rabbits to destroy cholesterol in limited quantity and their figures support the conclusion that when cholesterol is absorbed an equilibrium is set up between cholesterol in food, tissues, and excreta which with high cholesterol intake results in deposition of cholesterol in tissues.

The literature on response of blood cholesterol to fat feeding has recently been so well reviewed by Bloor (1) that this aspect of the problem need not be considered here. As stated above, Bloor's own data indicate that the general level of blood cholesterol in dogs 14 hours after the last meal is affected by fat intake. The relationship of this higher level of blood cholesterol to cholesterol deposition in tissues or from bile is a subject calling for further study of laboratory animals.

Our own data, as will be seen from the summary in Table II, indicate that the feeding of the cholesterol represented by a moderate number of egg yolks tends to produce a consistent, if slight, rise in the mean postabsorptive level of blood cholesterol in normal women. The less marked, or doubtful, indication of response to the feeding of cholesterol as such in butter may be due to the smaller number of blood samples from the period on the last named diet, or more probably, to a difference in absorption of the cholesterol. The intimate mixture of lecithin or lecithoprotein, fat, and cholesterol in egg yolk is not strictly comparable to crystalline cholesterol, which probably does not even stay in solution in butter fat throughout the alimentary tract. That

the effect of egg yolk as such is a question of considerable importance is evidenced by the increasing number of reports of development of arteriosclerosis in comparatively young diabetics incident to the recent use of high fat diets containing, as a rule, many eggs in the treatment of that disease.

The mean percentage of esterified cholesterol was slightly higher, *i.e.* 41.9 and 41.5 per cent, respectively, on egg yolk and on cholesterol as such than on the control diet, *i.e.* 39.4 per cent, probably not a great enough variation to be significant. But it is at least interesting to note that Bloor found a larger variation in the same direction in dogs on high fat diets, but not in rabbits on similar diets.

In general our observations of the mean percentages of esterified cholesterol in the blood of normal young women have given considerably lower figures than those found by Bloor in dogs. The mean values for a previous series of approximately 150 observations in this laboratory in which the colorimetric method was used with bloods of women on uncontrolled diets, were 43 per cent ester. The percentages of esterified cholesterol observed by Kaufmann and Mühlbock (12) in postabsorptive blood samples are sometimes in good agreement with those we have found. Sometimes, however, their subjects show a considerably higher percentage of ester. This may be due to differences in dietaries in Berlin and in California.

The degree of variability of blood cholesterol at the time of onset of menstruation does not appear to be greatly affected by a variation in cholesterol intake of the extent used in this study. Neither the data reported in this paper nor the unpublished data on uncontrolled diets, however, substantiate the earlier statement of Kaufmann and Mühlbock (13) to the effect that the menstrual drop in blood cholesterol is almost entirely a drop in free cholesterol. Both fractions seem to be involved; in some cases to an almost equal extent and in some cases the ester varies to a greater extent than the free cholesterol. The figures do suggest that the menstrual fall in ester is followed first by an increase in the free cholesterol, and then more slowly, by an increase in the ester fraction. This was true in even greater degree of the menstrual variations in ester cholesterol in some of our subjects on uncontrolled diets. It seems probable that free cholesterol is mobilized for some definite purpose during the menstrual period.

Dahlmos and Sole (14) and Degkwitz (15) have pointed out that cholesterol acts in the blood as a lyophobic colloid, while lecithin, as a lyophilic colloid has an antagonistic effect on membrane equilibrium. Our previous findings (2) indicate that the postabsorptive level of blood lecithin remains very nearly constant during the menstruation but that total fatty acid increases somewhat at about the time of the greatest fall in blood cholesterol. The investigators noted above found that when cholesterol was injected into the blood stream there was a hydremia with increased chloride in blood and urine, while injection of lecithin produced the opposite effect, *i.e.* imbibition of water by tissue with a decreased amount of water and chloride in the blood stream. This would suggest that variations in proportion of lecithin to cholesterol in the blood stream, such as we have observed during the menstrual period, may be associated with surface tension variations resulting in alterations in capillary permeability, and in tissue imbibition of water.

It has been impossible for us, with subjects who are actively engaged in college work, to make complete water balance experiments during menstruation. We have, however, kept daily weight records for some twenty students for periods of 1 month each. In approximately three-fourths of these there was no significant weight variation at the time of menstruation, but in the others there was a gain of 1 to 3 pounds shortly before the onset of menstruation with a sharp fall when menstruation began. And, interestingly enough, the students who showed the gain in weight almost all had histories of menstrual headaches or discomfort. Blood chloride determinations made in this laboratory on twelve subjects at 3 day intervals for 1 month (unpublished data, Okey and Foges) show a slight menstrual variation in postabsorptive blood chloride level in five cases only. The degree of variation is in the direction to be expected, but so small as to be statistically insignificant. Again, however, the variation was observed in the cases with the history of greatest menstrual discomfort.

All of this suggests the interesting possibility that menstrual congestion may be due to a water imbibition mechanism which results in increased tissue retention of water just before the onset of menstruation; and that this may in turn be due to a variation

in lipid ratios in blood and tissue. Our data are insufficient to justify a more definite statement. We feel, however, that the data here given indicate that, within the limits of the amounts to be found in the ordinary diet, cholesterol feeding has little effect on the menstrual variations.

#### SUMMARY

Data for free and total cholesterol of whole blood samples taken three times weekly are reported for four normal women who were placed successively, for periods of approximately 1 month each, on (1) a constant diet low in cholesterol, (2) one containing a moderate amount of cholesterol in egg yolk and liver, and (3) a diet low in food cholesterol to which a like amount of cholesterol as such was added.

The mean values for the month for both total and free cholesterol were consistently somewhat higher than for the control diet both when the diet containing egg yolk was given and when cholesterol as such was added to the control diet, but the diet containing the egg yolk produced the greater effect. Percentage of esterified cholesterol became slightly higher with increased cholesterol intake. The bearing of these findings upon the etiology of certain pathological conditions developing in patients fed therapeutic diets rich in egg yolk is discussed briefly.

The higher level of cholesterol intake was found to have very little, if any, effect upon menstrual variations in blood cholesterol in these particular subjects. A possible relationship between variations in lipid ratios in blood and tissues and the imbibition mechanism which produces menstrual congestion is suggested.

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## THE ANALYSIS OF WHOLE BLOOD

### IV. THE DETERMINATION OF GLUTATHIONE

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In the preceding papers of this series (1), procedures have been presented for the direct and accurate determination of glucose, of saccharoids, and of uric acid in complete filtrates from whole blood. In the present paper, this system of analysis is extended to include a simple direct colorimetric method for the determination of glutathione in filtrates from human blood. The procedure may be carried out in a few minutes upon as little as 5 cc. of the 1:10 tungstomolybdic acid filtrate, and is probably more accurate than the methods heretofore available for this determination.

Most of the procedures so far proposed for the determination of glutathione in blood have been based upon the reaction of glutathione with iodine. This reaction was first studied by Tunnicliffe (2), and has been used by Thompson and Voegtlin (3), and in modified form by Uyei (4), Blanchetière and Melon (5), Delaville and Kowarski (6), and Perlzweig and Delrue (7). Hess (8) determined glutathione in blood filtrates through application of Sullivan's specific reaction for cysteine (9) to blood filtrates after hydrolysis of the glutathione. Comparative determinations with the Okuda (10) iodine method showed agreement within about 10 per cent.

Hunter and Eagles (11) determined total glutathione in solution by a modification of the Folin-Looney (12) cystine method, but state that lack of specificity makes this method inapplicable to tissues. Braier and Marenzi (13) have applied the Folin-Marenzi (14) cystine method to the determination of glutathione in blood and tissues. These authors report that figures so obtained are lower than those obtained by Tunnicliffe's method (2),

and in fair agreement with those obtained by Gabbe's ferricyanide-iodine method (15).

In connection with his method for the determination of glutathione in blood and tissues which depends upon oxidation of the glutathione by ferricyanide, Mason (16) discusses the iodine procedure at length, and concludes that when applied to tissue extracts, this method may be quite inaccurate. Schelling (17) has very recently reported application of the Mason method to the determination of glutathione in blood. Woodward and Fry (18) were unable to employ the Mason method successfully in this same connection, because of the very rapid loss (through oxidation) of the glutathione from the tungstic acid filtrates. They state that it required 40 minutes to obtain enough filtrate to make a determination, and that the loss of glutathione during this period is quite considerable. It is to be regretted that these authors did not, through the use of the centrifuge, or of larger volumes of blood, secure filtrates more promptly so that comparative figures with their iodine procedure might have been reported.

As the Mason method appears, from the evidence so far available, to be more specific for glutathione than the iodine methods, and apparently gives figures for blood glutathione as low as those obtained by other procedures so far available, we have selected results by this method for comparison with those obtained by our new procedure.

The method which we wish to propose is based upon the fact that the arsenophosphotungstic acid reagent for uric acid proposed by one of us (19) is reduced by glutathione in acid solution with the formation of a blue color which is proportional to the quantity of glutathione present. In constructing this method, we were led to test the behavior of glutathione with the arsenophosphotungstic acid solution because of the interesting results obtained when glutathione and a small quantity of copper sulfate were added to a phosphomolybdic acid color reagent (20). The cupric ion was reduced almost immediately in the cold (an observation previously recorded by Hopkins (21)) and this in turn instantly reduced the phosphomolybdic acid reagent with the production of the characteristic blue color. While constructing a method for the determination of glutathione based upon this reaction, we found that although thioneine, even in large quantity in pure solution, gave

only a trace of color with copper sulfate and phosphomolybdic acid, the oxidation of the thioneine was catalyzed by glutathione to a remarkable degree, so that in presence of thioneine the apparent glutathione was markedly increased.<sup>1</sup> This interaction of glutathione and thioneine, whereby the latter may be oxidized only in presence of the former, is especially interesting in view of the finding reported by Woodward and Fry (18) that oxidation of thioneine by ferricyanide and by iodine is very greatly increased in presence of glutathione. In view of these three examples of the apparent catalytic interrelationship *in vitro* of the two compounds, it is perhaps not going too far to suggest that a similar relationship may exist *in vivo* in the red blood cell, which has been shown to contain both of these compounds.

We shall carry out further studies with the copper-phosphomolybdic acid reagent, but we abandoned its use in connection with determination of glutathione in blood because of interference due to thioneine. In the method now to be described, the interference due to thioneine, while detectable with relatively large amounts of thioneine, is so slight as to be of no practical significance in the analysis of human blood.

Before describing the exact technique for the new method, it may be well to comment upon some points in connection with it. The reduction of the arsenophosphotungstic acid reagent by the glutathione is carried out in the presence of a large excess of sodium acetate. Addition of this salt lowers markedly the acidity of the reagent, and provides a buffer against the moderate amounts of acid present in the blood filtrates. Under even optimal conditions of reagent and acetate the depth of color yielded by the glutathione content of blood filtrates is not great, and many would find it difficult to read the color satisfactorily if the blood has been diluted 1:10. Therefore, in employing the method in this way, it is desirable to make use of filtrates from blood samples which have been diluted only 1:5. Early during the work it was found,<sup>2</sup> however, that the presence of even small amounts of sulfite dur-

<sup>1</sup> When thioneine is present, a precipitate may form upon addition of the copper sulfate solution. Enough hydrochloric acid was added to dissolve this precipitate.

<sup>2</sup> That  $\text{NaHSO}_2$  increases the color given by this reaction was pointed out to us by Miss Gladys Fashena of the Cornell University Medical College.

ing the reaction would markedly increase the reducing power of glutathione as indicated by the amount of color produced. Thus, in presence of 3 drops of 2 per cent sodium bisulfite solution in a volume of slightly over 7 cc., the color yielded within 10 minutes by a given amount of glutathione is increased about 75 per cent as compared with that obtained in absence of the bisulfite. As will be shown later, only a negligible amount of the increased color in presence of sulfite is due to color produced by the sulfite itself. Neither can the marked effect of the sulfite be explained on the basis that we are, through its presence, reducing oxidized glutathione. The increase in color following addition of bisulfite was obtained with pure solutions of glutathione (Eastman Kodak Company) in which there was at least 97 per cent reduced glutathione as indicated by titration with iodine. A practically identical increase in color occurs with fresh blood filtrates in the presence of bisulfite. If the procedure with and without bisulfite is applied to 1:5 filtrates, figures for glutathione by the method obtained without bisulfite average about 1.6 mg. per 100 cc. of blood lower than when bisulfite is employed (Table I). These findings do not, however, warrant the conclusion that the method without sulfite is more accurate, since studies of the recovery of glutathione added to blood (Table III) show a greater loss when the method is used without sulfite. The percentage difference in recovery of added glutathione by the two methods almost exactly coincides with the percentage difference between the figures obtained upon the blood filtrates with and without sulfite. Thus, we are led to conclude that the use of sulfite yields figures which are more accurate than those obtained without its use. Since sulfite has been employed for the reduction of cystine to cysteine (Folin and Marenzi (14)), it appeared that when sulfite is employed in our method, the figures thus obtained may include some oxidized glutathione originally present in blood filtrates. If so, the quantity included is too small to be demonstrated by any procedure now available for a study of this question. We are studying further the possible usefulness of sulfite in the determination of oxidized glutathione but we may conclude at the present time that for the most accurate results sulfite should be employed.

In connection with the determination of glutathione in blood it should be remembered that the glutathione is confined largely,

if not entirely, to the corpuscles. It is therefore essential, if results which are too low are to be avoided, that the blood analysed shall be entirely free from clots. Excessive amounts of oxalate in blood retard color development. Not more than 2 to 3 mg. of sodium or potassium oxalate per cc. of blood should be used.

Below are given the reagents employed and the technique of the new method.

### *Reagents*

*Arsenophosphotungstic Acid (19)*—100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter. For use, a portion of this reagent is diluted with an equal volume of water and placed in a burette in which the number of drops delivered per cc. has been determined. About 0.3 cc. of the diluted reagent is used for the determination.

*Sodium Acetate*—50 per cent solution is used. With the aid of heat dissolve 250 gm. of sodium acetate in about 250 cc. of water, dilute the solution to about 500 cc., mix, cool, dilute to volume, and filter.

### *Standard Solution*

*Stock Solution*—Dissolve 70 mg. of glutathione<sup>3</sup> in 100 cc. of 0.1 N HCl. This solution will keep with no detectable change for at least 1 month in a refrigerator at 4°.

*Working Standard*—5 cc. of the stock solution are diluted to 100 cc. so that 5 cc. of the diluted solution contain 0.175 mg. of glutathione, equivalent to 35 mg. per cent of glutathione in the original blood.<sup>4</sup>

<sup>3</sup> For preparation of standard solutions for use in our method and with the Mason method, we have used glutathione obtained from the Eastman Kodak Company.

<sup>4</sup> This standard will be satisfactory for use with most bloods which may be encountered, provided they have been properly mixed and were not clotted. If the determination indicates less than 25 or more than 60 mg. of glutathione per 100 cc. of blood, it is preferable to repeat the determination, a lower standard or less of the filtrate being used.

*2 Per Cent Sodium Bisulfite*—About 0.2 cc. is measured from a dropping bottle or from a burette. The color development is retarded when this solution is not fresh. A 2 per cent solution kept at room temperature cannot be counted on longer than 1 week, but will keep for at least 2 weeks if kept in the ice box at 4°. A 10 per cent solution will keep at room temperature for a month, and dilutions can be made weekly.

#### *Procedure*

1:10 tungstomolybdic acid filtrates (22) are used. Autooxidation of glutathione in the filtrates can be prevented by acidifying immediately with 1 drop of 0.62 N  $\text{H}_2\text{SO}_4$  per 10 cc. of filtrate. The decrease in glutathione in unacidified filtrates at room temperatures is not rapid, amounting to only about 3 per cent an hour.

To 5 cc. of the blood filtrate in a test-tube and to 5 cc. of the standard solution, 2 cc. of the sodium acetate solution are added, followed by about 0.2 cc. of 2 per cent sodium bisulfite solution and about 0.3 cc. of the diluted uric acid reagent. The solutions are mixed by inversion and read in the colorimeter after 10 and before 30 minutes. The color development is slow, so the standard solution must be run side by side with the unknown solutions. We have found it convenient to use the white glass colorimeter cups made by the Klett Manufacturing Company, which require only about 3.5 cc. of liquid to fill them.

The blank in the new method is equal to about 8 per cent of the color of a 50 mg. per cent standard. This does not interfere with satisfactory proportionality. With a reagent that has stood for a few weeks practically perfect proportionality is obtained when a standard containing 0.35 mg. of glutathione equal to 70 mg. per cent is read against one containing 0.175 mg. equal to 35 mg. per cent of glutathione. If a blood contains more than 70 mg. per cent of glutathione, the determination should be repeated with a smaller aliquot of filtrate. With *freshly prepared reagents* 50 mg. per cent of glutathione are read against 35 mg. per cent with a maximal error of 6 per cent, but proportionality over a wide range is poor and unless the reagent is some weeks old the reading between standard and unknown should not vary by more than 25 to 30 per cent. Interference due to thioneine is very slight. A solution corresponding to 20 mg. per cent of thioneine in blood gives only

slightly more color than is obtained with a blank. 20 mg. per cent of thioneine added to 25 mg. per cent of glutathione increase the color about 6 per cent; when added to 50 mg. per cent of glutathione, the color is decreased about 4 per cent, and added to 40 mg. per cent of glutathione, the color is depressed 1 to 2 per cent.

Comparative determinations by Mason's (16) method and the new method with and without sulfite<sup>5</sup> on 1:5 and 1:10 blood filtrates have been made. Mason's method has been applied to 2.5 cc. of 1:5 or 5 cc. of 1:10 filtrates; one-fifth the amount of reagents was used and the mixture was diluted to a final volume of 10 cc. Glutathione was used as the standard. As the sample of Congo red in the laboratory indicated a pH below 5.9, brom-cresol purple has been used. An aliquot of filtrate was titrated with 0.1 N NaOH, and after addition of the buffer, compared with a standard buffer solution. Folin's gum ghatti solution (23) was substituted for the gum arabic solution. First Folin's picrate paper filter (24) and then a yellow glass filter (Klett Manufacturing Company) were found convenient aids in making the color comparison. Several formaldehyde blanks, with the same amount of material as employed for the determination, were found to be negative in accord with the findings of Mason (16) and Schelling (17), so that determination of a blank was omitted.

In Table I are recorded comparative values for glutathione obtained from single and pooled samples of normal human blood. The values obtained with and without sulfite vary together, the average for the method with sulfite being 1.6 mg. per cent higher than for the method without sulfite. As is seen in Table II, the better recovery of added glutathione with sulfite indicates that the figures with this technique are probably the more correct ones. There is general agreement between the figures obtained by Mason's method and the new method, but the average value of ten determinations on nine different samples of blood for Mason's

<sup>5</sup> The method without sulfite is carried out as follows: To 5 cc. of the 1:5 tungstomolybdic acid blood filtrate in a test-tube and to 5 cc. of the standard solution containing 0.35 mg. in 5 cc. (10 cc. of the stock solution diluted to 100 cc.) add 2 cc. of 50 per cent sodium acetate solution and 0.2 to 0.24 cc. of Benedict's uric acid reagent (not diluted) measured in drops from a burette. Mix by inversion and read in the colorimeter after 5 and before 30 minutes.

method is 7.9 mg. per cent higher than with the method without sulfite and 6.3 mg. per cent higher than with the method with sulfite.

TABLE I  
*Glutathione (Mg. Per Cent) in Single and Pooled Samples of Normal Human Blood\**

Sample No	Tungstomolybdic acid filtrate				
	1:5†			1:10	
	Mason method on 2.5 cc.	Method with bisulfite on 2.5 cc.	Method without bisulfite on 5 cc.	Mason method on 5 cc.	Method with bisulfite on 5 cc.
1	38	30	28		31
2	36	31	29		
3	38	33	31		
3	37	35	34		
4	48	35	35		
5	44	36	35		39
6	44	37	35		
7	48	40	39		40
8	53	43	42		
9	42	44	42		
Average of 10 determinations...	42 8	36 4	35 0		
10		32		38	33
11		38	35	41	39
12		38		47	41
13		44		54	45
9		47		58	48
Average of 5 determinations...		39 8		47.6	41.2

\* In these determinations, 0.2 cc. of dilute reagent was employed instead of the 0.3 cc. now used. This change in quantity of reagent was made because after this work had been completed fresh samples of reagent were found which did not give maximal color development with the smaller quantity.

† These filtrates were prepared by laking blood with 3 volumes of water and adding  $\frac{1}{2}$  volume of double strength tungstomolybdic and sulfuric acids. Double strength tungstomolybdic acid must be kept at about 37° to prevent crystallization.

Eight comparative determinations made by the new method on 1:5 and 1:10 filtrates of the same blood gave values averaging 3.4 per cent higher on the 1:10 filtrate, with a maximal difference of 8 per cent.

The range in values for normal blood obtained by the method with bisulfite on 1:5 and 1:10 filtrates is 30 to 48 mg. per cent, and by Mason's method on 1:5 and 1:10 filtrates 36 to 58 mg. per cent. These latter values are in marked contrast to figures of 14.7 to 38.8 mg. per cent obtained for normal blood with Mason's method by Schelling (17). Possible explanations of this discrepancy are presented later.

TABLE II  
*Glutathione and Hemoglobin in Human Blood*

Blood sample No.	Hemoglobin	Glutathione	
		Mason method	New method (with bisulfite)
	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Hospital, No. 7.....	94	31	29
Normal, No. 3.....	93	37	35
Hospital, No. 6.....	92	42	34
Normal, No. 3.....	92	38	33
Hospital, No. 13.....	86	29	29
“ “ 5.....	83	30	27
“ “ 10.....	78	36	26
“ “ 9.....	73	34	26
“ “ 8.....	65	29	25
“ “ 4.....	62	30	23

The average value obtained by the three methods for eleven bloods from hospital cases is 26 to 29 per cent lower than that obtained from ten normal human bloods. This is at least partly explained by clotting in many of the hospital samples and a loss of red cells due to inadequate mixing of the samples on pooling. An excess of oxalate was found to interfere with each of the three methods, but as this interference is much more marked with the Mason than with the new method, it cannot account for the uniform difference between the two series by the different methods. As the bloods from hospital cases stood at room temperature for some time before being delivered to us, aliquots of two fresh samples of blood were permitted to stand at room temperature for

6.5 hours. Glutathione values obtained by the three methods after standing were the same as those obtained on the blood when fresh.

Mason has noted the retarding effect of large amounts of salts on his reaction. We have found that 15 mg. of potassium oxalate per cc. of blood added to two samples of blood gave decreases of 31 and 35 per cent with the Mason method, 14 and 11 per cent with the bisulfite method, and 9 per cent in both instances with the method without bisulfite. A marked depression also occurred on adding 10 mg. of potassium oxalate per cc. of blood. The equivalent of 2 mg. of oxalate per cc. of blood added to the standard solution produced a marked depression with the Mason method but did not affect the new method. Oxalate added to the blood, however, appears in the filtrate only when added in excess, and then in small part. Eight filtrates of highly oxalated bloods from hospital cases were tested for oxalate by adjusting the pH with acetic acid and adding  $\text{CaCl}_2$ . An abundant precipitate was formed in one filtrate, slight precipitates in three, and no precipitate at all in the remaining four filtrates. Sheep blood was therefore shaken with varying amounts of oxalate and placed in the refrigerator for 1 hour. 2 mg. of oxalate per cc. added to blood were not detectable in the filtrate and did not affect any of the three methods. When 4 mg. of oxalate were added per cc. of blood, only a trace of precipitate was formed in the filtrate on the addition of calcium; the new method was not affected, but inconsistent results averaging 4 per cent lower were obtained with Mason's method. With 8 mg. of oxalate added per cc. of blood less precipitate was formed in the filtrate on the addition of calcium than in the control in pure solution containing oxalate equivalent to 1 mg. of oxalate per cc. of blood. The figures by the Mason method were depressed 11 per cent, and by the bisulfite method 5 per cent. Congo red was used as indicator in the Mason method in these determinations. While excess of oxalate therefore must be avoided, 2 mg. of oxalate per cc. will not affect any of the three methods and are in excess of the amount required to prevent coagulation.

Several workers (3, 4, 25, 26) have found glutathione to be present in the corpuscles and not in the serum of animal bloods, while Turner (27) has apparently assumed a negligible glutathione content for human serum. The direct correlation in human blood

between hemoglobin determined by Newcomer's method (28) and glutathione determined by the new method is shown in Table II. Strangely enough, there appears to be no direct relation between

TABLE III

*Recovery of 30 Mg. Per Cent Glutathione Added to Human Blood in Laking Water*

Tungstomolybdic acid filtrate												
Blood	1:5						1:10					
	Method without bisulfite		Method with bisulfite		Mason method		Method with bisulfite		Mason method		Remarks	
	Original glutathione content of blood	Added glutathione recovered	Original glutathione content of blood	Added glutathione recovered	Original glutathione content of blood	Added glutathione recovered	Original glutathione content of blood	Added glutathione recovered	Original glutathione content of blood	Added glutathione recovered		
	mg. per cent	per cent	mg. per cent	per cent	mg. per cent	per cent	mg. per cent	per cent	mg. per cent	per cent		
Clinic. ....	28.5	95.4	29.4	100	35.1	102.3						
Normal. ....	35.0	91.6	37.4	94.7	44.1	97.0						
" .....	34.5	86.0	34.8	85.4	47.9	90.0						
Clinic. ....	24.8	93.0	26.2	99.4	36.4	100.0	26.4	99.0			Laked with 8 vols. H <sub>2</sub> O	
Hospital. ....	24.7	81.4	26.4	87.0	33.9	79.3	28.1	97.0			" "	
Normal. ....							29.6	98.5			Laked with 7 vols. H <sub>2</sub> O	
" .....							40.8	94.0			" "	
" .....							40.0	93.4	49.8	91.3	" "	
" .....							34.6	95.6	43.8	90.6	" "	
Average recoveries. ....		89.5		93.3		93.7		96.3		91.0		

hemoglobin and the glutathione values obtained by the Mason method. Determinations made upon 1:5 filtrates of two samples of human blood plasma indicated the presence of some glutathione. The glutathione content of plasma is being further studied.

Recoveries of glutathione added to blood are shown in Table III. Standard glutathione solution was neutralized and added to the blood in the laking water. Recoveries are uniformly good with the 1:10 filtrates, but two of five recoveries with 1:5 filtrates are poor. Schelling (17), however, reports consistently good recoveries with 1:5 filtrates with Mason's method.

Three samples of sheep blood gave higher figures with the new method than with Mason's method. We are studying this question further, and for the present advise the use of the new method only when applied to human blood.

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*Note*—Uric acid yields a slight color in this method, but in the presence of the amount of glutathione contained in blood filtrates, the effect is negligible. Thus 5 mg. per cent of uric acid produce no effect; 10 to 20 mg. per cent of uric acid may increase the apparent glutathione by about 1 mg. per cent.

## THE ISOELECTRIC POINT OF INSULIN

### ELECTRICAL PROPERTIES OF ADSORBED AND CRYSTALLINE INSULIN\*

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Chemical researches on the crystalline protein first obtained from commercial insulin preparations by Abel (1) in 1926 have left no doubt that this substance is identical with the hormone in its purest stable form and represents a definite chemical entity, though it may be well to remember that this term should be applied in protein chemistry with certain reservations. The recent experiences of Sørensen (2) with crystalline egg albumin, as well as certain suggestions made by Freudenberg and Dirscherl (3) in regard to insulin itself, are of interest in this connection. The isoelectric point of crystalline insulin has not been determined hitherto in more than an approximate fashion, though the literature contains many references to measurements on more or less purified amorphous insulin preparations. Abel and coworkers (4) noted that the pH of a solution from which insulin crystallized in satisfactory yields was 5.60 to 5.65. These solutions contained ammonia, pyridine, brucine, and acetic acid in high concentration. Since such a medium is highly instrumental in holding insulin in supersaturated solution and in favoring the slow deposition of crystals, the influence of these different constituents on the solubility of insulin must be considerable. The conditions in the presence of materials like pyridine and brucine can hardly be compared to those prevailing in dilute univalent buffer systems. It seemed of theoretical as well as of practical interest to determine the iso-

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electric point of crystalline insulin by electrophoresis and to compare the results with those obtained by solubility measurements and nephelometric determination of the point of maximum precipitation.

### *Methods*

*Insulin*—The insulin used in the experiments was obtained in crystallized form by the brucine method of Abel. For the electrophoresis experiments and the solubility measurements a sample was used which had been recrystallized three times without addition of brucine. The preparation used for the flocculation experiment was from the same source but recrystallized only once.

*pH Measurements*—The pH measurements were carried out at 25°, by the method of Rosebury (5), with a film glass electrode of the type described by MacInnes (6).

*Electrophoresis*—The electrophoretic method employed in this work is based on direct measurement of the electric mobility of microscopically visible quartz particles coated with an adsorbed film of protein (7). The justification for the employment of this method depends upon the experimental fact that the electric mobility of quartz particles covered with a film of egg albumin or of serum albumin is, within the limits of error, practically the same as that of the same proteins studied under the same conditions by the moving boundary method of Tiselius (8). It would perhaps be better to use the moving boundary method on insulin, but because of its low solubility in the isoelectric zone and unavailability in large quantities in the pure state, the microscopic method is preferable. It is assumed that the quartz particles covered with a film of adsorbed insulin possess the electric mobility of the protein in the dissolved state, as is the case with egg albumin and serum albumin. It was possible by this method to study not only the mobilities of insulin-covered quartz particles but also those of amorphous particles of insulin and of insulin crystals.

The quartz used in the experiment was boiled with hydrochloric acid, then treated with hot chromic acid-sulfuric acid mixture, and finally washed with distilled water for several days.

For the preparation of the insulin-coated quartz suspensions a suitable amount of insulin was dissolved in 0.4 M sodium acetate solution and a small volume of quartz suspension was added. In

this way the quartz surfaces remained in contact with a relatively concentrated insulin solution for about 10 minutes. Then the appropriate amounts of acetic acid and water were added to bring the pH to the required value and to make the final concentration of acetate ion  $m/30$ . The measurements were carried out at room temperatures varying between 24–27°. No preservative was needed.

*Solubility Measurements*—Insulin crystals and  $m/30$  acetate buffer of varying pH were placed in Pyrex bottles, sealed with paraffin, and rotated or shaken in a thermostat at  $25^\circ \pm 0.01^\circ$ . The amount of insulin and the volume of the solution were varied according to the expected solubility. A small amount of toluene was added as a preserving agent. The time of shaking was 48 hours. Before the solution was filtered, care was taken to assure, by microscopic inspection, the presence of well defined crystals in the solid phase, for it was occasionally observed that some amorphous material (probably formed by slight denaturation under the combined action of shaking and of toluene) was suspended in the solution at the end of the shaking period. Solubility values in the neighborhood of the isoelectric point did not increase between 24 and 48 hours. Filtration was effected through a very small (8 mm.) asbestos filter, in which losses due to adsorption were probably negligible compared with adsorption by the glassware. Suitable volumes of the clear filtrate were analyzed in duplicate for nitrogen by the micro-Kjeldahl method of Pregl. Blank determinations were carried out on large volumes of the buffer solutions used, after they had been subjected to the same procedure as the solutions containing insulin. No nitrogen could be detected in the buffer solutions.

*Nephelometric Determinations of Point of Maximum Flocculation*—Insulin was dissolved in 0.4  $m$  acetate and precipitated at varying activities of hydrogen ion by the addition of the appropriate amount of acetic acid and water. The insulin concentration in respect to the final volume of the solution was 1:10,000. The buffer concentration was  $m/30$  in respect to sodium acetate. At an insulin concentration of 1:10,000 the suspensions obtained in the neighborhood of the isoelectric point remained stable for about 2 hours. Doubling this concentration resulted in microscopic flocculation within 5 to 7 minutes. A measure of the rela-

tive turbidities of the insulin suspensions was obtained by comparing them in a Kober nephelometer with standards of isoelectric casein suspensions stabilized with gum arabic. The relative turbidities of the standards were found to conform with their relative concentration within 5 per cent. In order to test the experimental procedure measurements were made on non-stabilized casein suspensions obtained by precipitating casein dissolved in sodium acetate at varying values of pH by the addition of acetic acid. At a casein concentration of 1:10,000 the turbidity values were constant between pH 4.4 and 5.0 with a sharp drop on either side of that range, suggesting an isoelectric point for casein in the neighborhood of 4.7, in agreement with the data in the literature. With a lower casein concentration (1:50,000) the zone of constant turbidity around the isoelectric point became narrower, but the reproducibility of the turbidity values became less satisfactory. With higher casein concentration (1:5,000) macroscopic flocculation took place within a very short time.

### *Results*

*Adsorption of Insulin on Quartz*—In common with most proteins insulin is adsorbed by quartz particles, with profound alteration of their surfaces, and consequently of their electrokinetic properties. A certain minimum concentration of insulin in the solution in which the quartz particles are suspended is required to reach a limiting value of the effect of the protein on the electric mobility, which is taken to represent complete coating of the particles with the protein. In order to obtain the exact value of this minimum concentration two series of experiments were carried out on either side of the isoelectric point; in these, quartz particles were suspended in solutions of insulin in 0.4 M acetate of varying insulin concentrations, and the pH brought to 4.0 and 6.0 respectively by the addition of acetic acid. The final sodium acetate concentration was M/30. Fig. 1 gives the relation between the electric mobilities of the particles and the insulin concentration. The ordinate values are in  $\mu$  per second per volt per cm.; the abscissæ in weight fractions of insulin in the aqueous medium referring to the final concentration of insulin in the solution after addition of acetic acid and water. That is to say, the concentration of insulin to which the quartz particles were originally exposed was higher

than the final concentration and can be obtained by multiplying the figures on the abscissa scale by 12.

The quartz particles change their surface charge in the presence of the merest traces of insulin. The saturation point is practically reached when the insulin concentration is about 1:20,000. This experiment yielded the information that between pH 4.0 and pH

TABLE I

*Interpolated Values of Electric Mobility of Insulin (Adsorbed) in S/30 Sodium Acetate Buffer ( $V_m$  in Cm. per Second per Volt per Cm.  $\times 10^4$ )*

pH.....	4.0	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0
$V_m$ ....	+1.58	+1.48	+1.29	+0.93	+0.56	+0.19	-0.16	-0.51	-0.83	-1.12

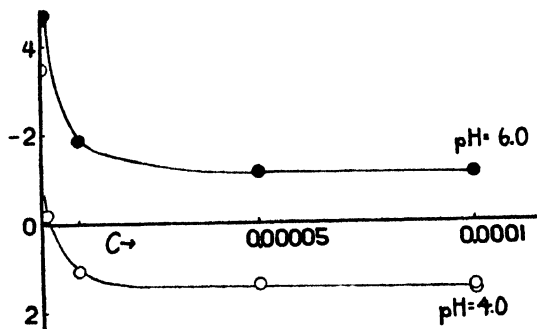


FIG. 1. The adsorption of insulin by quartz. The ordinate units are in  $\mu$  per second per volt per cm.; the abscissa units are in weight fraction of insulin in buffer.  $C$  indicates concentration. The quartz particles change their surfaces in the presence of the merest trace of insulin, saturation being practically reached when the insulin concentration is about 1:20,000. This experiment yielded the information that between pH 4.0 and pH 6.0, adsorbed insulin probably completely covered the quartz particles.

6.0 adsorbed insulin probably completely covered the quartz particles under the conditions adopted.

*Electric Mobility of Adsorbed and Amorphous Insulin*—Curve I in Fig. 2, A shows the influence of pH on the mobility of quartz particles presumably completely covered with insulin. The suspensions were prepared as indicated under "Methods." The final concentration of insulin in these experiments was 1:10,000. In the neighborhood of the isoelectric point a part of the insulin was

precipitated under these conditions in the form of amorphous aggregates which could be distinguished under the microscope from the quartz particles by the difference in their refractive power. These amorphous aggregates move in the electric field with the same velocities as the quartz particles. From these measurements it appears that the point of zero mobility lies between pH 5.3 and pH 5.35, and according to the assumptions stated in the beginning of the paper the isoelectric point of dissolved insulin is probably to

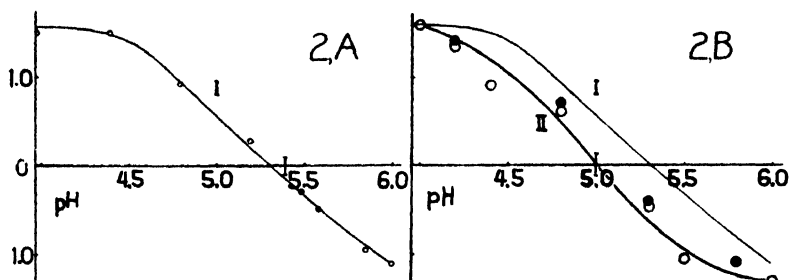


FIG. 2, A. The electric mobility of quartz particles covered with insulin in  $M/30$  acetate buffers. The isoelectric point is between pH 5.30 and pH 5.35. The same data were obtained with particles of amorphous insulin. The ordinate units are in  $\mu$  per second per volt per cm.

FIG. 2, B. The smooth curve (Curve I) gives the electric mobilities of adsorbed or of amorphous insulin. The lower curve (Curve II) gives the mobilities of insulin crystals or crystal fragments in the same medium. Curve II has been roughly fitted to the open circles (mobilities of crystals suspended in  $M/30$  acetate buffer). For significance of the open and solid circles consult the text. The ordinate units are in  $\mu$  per second per volt per cm.

The readings below the zero line denote negative mobilities.

be found in that range. For convenience, the interpolated values of the motilities of insulin adsorbed on quartz are given in Table I.

*Electric Mobility of Insulin Crystals*—The electric mobilities of insulin crystals suspended in  $M/30$  acetate buffers of varying pH were also studied. The crystals were kept in contact with the buffers for about 10 minutes before the measurement was made. Crystal fragments obtained by grinding the crystals in an agate mortar showed essentially the same mobility as the intact crystals. The mobilities observed are represented by the open circles in Fig. 2, B. The smooth curve (Curve II) drawn through these

points cuts the pH scale on a definitely lower level, in the neighborhood of pH 5.0. Curve I is given for comparison and represents the mobilities of adsorbed or amorphous insulin. The differences between the two curves is well outside of the range of experimental error. Measurements were also made on insulin crystals suspended in insulin-containing acetate buffers which had been made up in the same way as the insulin-coated quartz suspension used in the measurements described above, and brought to the required pH *before* addition of the crystals. The mobilities found in these measurements are given by the solid circles in Fig. 2, *B*. In the neighborhood of the isoelectric point a part of the insulin, as mentioned above, is precipitated under these conditions, but such solutions probably remain in a state of supersaturation with respect to insulin for considerable time after the precipitation, as will be pointed out later. It is therefore to be expected that a certain amount of insulin will be deposited on the surface of crystals left in contact with such solutions. As a matter of fact, it was found that the mobilities of crystals under these conditions do not deviate appreciably from the values for crystals suspended in the pure buffer solutions presumably incompletely saturated with insulin. If deposition of insulin from the solutions considered as supersaturated has taken place on the surface of the crystals, it does not seem to have influenced their surface charge to any greater extent than those of crystals not in equilibrium with a saturated solution, as can be seen from Fig. 2, *B*.

*Solubility of Insulin in  $m/30$  Acetate Buffers*—The results of the solubility determinations are represented by Curve I in Fig. 3. Over the broad range pH 4.6 to 6.5 the solubility is very low. The solubility values between pH 4.8 and 6.2 fluctuate between 3.1 and 4.0 mg. per liter. At pH 6.0 a value of 5.3 mg. was found, probably due to experimental error. With increasing solubilities at both sides of this pH range the reproducibility was not very satisfactory. We have not tested the possibility that the solubilities are dependent on the amount of solid phase present, nor that at pH values where the solubility increases the solution had failed to attain equilibrium with the solid phase within the time allowed for saturation. Our limited supply of highly purified material prevented further inquiry into these points. It may be mentioned in this connection that the recovery of crystalline material from

those parts of the solution which were not used for the nitrogen determination was not as good as expected, so that possibly some denaturation may have taken place. It is difficult to decide from the present data whether the shape of the curve is strictly symmetrical, as is the case with ideal ampholytes. These circumstances, in conjunction with the uncertainty of the absolute solubility values in the regions where the solubility increases, throw doubt on the application of the mass law to the evaluation of the isoelectric point. If a smooth curve be drawn through the experimental points, and the isoelectric point be taken as the mean of

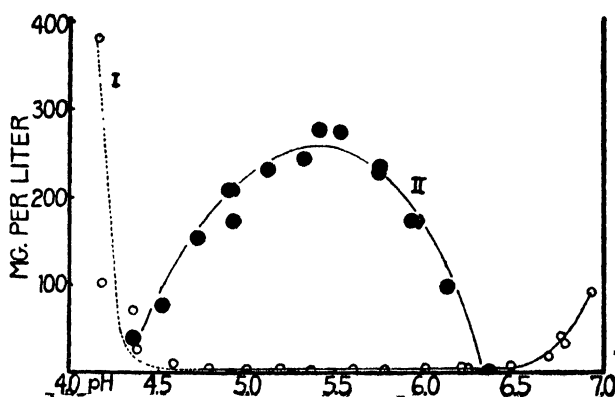


FIG. 3. The open circles (Curve I) represent the solubilities of crystalline insulin in  $M/30$  acetate buffers in mg. per liter. The dotted part of the solubility curve indicates uncertain course. The solid circles (Curve II) represent relative turbidities.

two points of equal solubility on that curve, values between 5.55 and 5.60 are obtained. However, the positions of this so called isoelectric point would, when the solubility curve is not symmetrical, depend on the level of solubility chosen.

*Point of Maximum Flocculation*—Readings on insulin suspensions prepared as indicated under "Methods" were taken from the 3rd to the 12th minute after the addition of the acid to the solution of insulin in sodium acetate. In the neighborhood of the isoelectric point (pH 4.8 to 6.0) the turbidity values remained practically constant after the 5th minute. At higher or lower pH values a slight increase could be noted during the period of observation. The (relative) turbidity values determined between the 10th and the

12th minutes were used for constructing Curve II in Fig. 3. No aggregation of particles visible to the naked eye was observed during this period. The turbidity values obtained for suspensions prepared under identical conditions were reproducible within about 10 per cent, though in most cases the agreement was much better. The comparatively large limits of error make it difficult to ascribe a very definite value to the point of optimum flocculation. A smooth symmetrical curve drawn through the experimental points indicates a maximum of flocculation at approximately pH 5.4, which is in fair agreement with the value pH 5.3 to 5.35 found for the isoelectric point by mobility measurements.

#### DISCUSSION

Besides giving the required information as to the isoelectric point of insulin in  $M/30$  acetate buffer the experiments reported contribute a few observations of general interest. The difference observed between the mobilities of adsorbed or amorphous insulin and of crystals suspended in the same medium shows that the surface charge of the amorphous particle differs from that of the surface composed of the limits of the crystal lattice. The isoelectric point of the crystal deviates by 0.3 pH units from that of insulin-coated quartz particles or amorphous insulin. Since the behavior of the latter reflects in all probability that of the dissolved protein, a smaller number of positively charged groups is available on the crystal surface than on the freely dispersed molecule over the pH range under consideration. In this respect the behavior of insulin crystals resembles the behavior of crystals of the less soluble amino acids, tyrosine, cystine, and aspartic acid. One of us (9) has previously observed that the isoelectric point determined by electric mobility of crystals of these amino acids suspended in dilute hydrochloric acid and in buffers is the same, namely about pH 2.3, and bears no relationship to the isoelectric point of these amino acids in the dissolved state as determined by the magnitude of their dissociation constants. The behavior of crystals of the above amino acids in protein solutions has been compared with that of protein-covered quartz particles and other inert surfaces. The surfaces of the amino acid crystals adsorbed gelatin just as inert surfaces do, the electric mobilities of both being identical.<sup>1</sup>

<sup>1</sup> Unpublished data.

The insulin crystals have here been examined in dilute solutions of insulin. The surface of the insulin crystals could be (1) entirely made up of the limits of the crystal lattice, (2) a surface completely covered by adsorbed protein, (3) a combination of (1) and (2), the surface being partially made up of crystal lattice and of adsorbed protein. Fig. 1 demonstrates that very minute traces of insulin changed the electrical mobility of quartz particles. If this fact be taken, together with the fact that the ampholyte surfaces of the amino acids mentioned before seem to adsorb protein quite readily, it follows that only when every molecule of insulin colliding with the lattice enters the lattice at precisely the same rate as the rate of collision, can case (1) be realized. It seems unlikely that this ideal case can occur, for in the most acid of the solutions studied (at about pH 4) where insulin becomes soluble, the mobilities of both amorphous and crystal surfaces approach one another. In an analogous fashion a charge reversal of the amino acid crystals is observed in a region where these amino acids become soluble. In other words, here, the crystal surface loses its integrity and acts as an amorphous surface. The simplest explanation would be that the insulin crystals adsorbed sufficient insulin from the solution to cover their surfaces in just the same way as the surfaces of inert particles are covered, the area involved being dependent on the amount of insulin in solution (see Fig. 1). Variations in the proportion of crystal surface to amorphous surface would thus account for the differences between the mobilities of adsorbed insulin and insulin crystals in the isoelectric region. We admit that the experimental evidence adduced in this work is insufficient to permit any far reaching conclusions to be drawn with regard to the differences between the surface charges of crystals and of amorphous particles of amphoteric substances. A careful study of these phenomena on a crystalline protein of relatively low solubility in the isoelectric range, but more easily available than crystalline insulin, seems highly desirable.

The possibility has also to be considered that the crystals used in the electrophoresis experiments did not actually represent the isoelectric protein in a strict sense. The pH of the solutions from which the crystals of insulin employed in these experiments were obtained was not measured. However, it could not have been very far removed from the pH of the solution containing ammonia,

pyridine, and acetic acid in the proportions employed for the recrystallization. The pH of such solutions was found to lie between 6.0 and 6.1. The insulin crystals may therefore have contained small amounts of base, presumably bound to the acidic groups of the protein. If these groups on the surface of the crystals were fully dissociated at the true isoelectric point of the protein, then the number of positively and negatively charged groups on the surface would be determined only by the pH of the solution; in other words the crystals would show the same isoelectric point as freely dispersed protein molecules. If, on the other hand, the crystal surface retains the bound base, then the number of groups able to carry a negative charge would be diminished; that is, the crystal should assume a positive charge at the (true) isoelectric point. Since it was found that the crystals are highly negatively charged at this point, the discrepancy discussed above is probably not caused by differences in the *chemical* composition of the amorphous and the crystalline surface.

Another point of interest is the difference between the isoelectric point as determined from the solubility data and from the turbidity determinations. Comparison of the solubility and turbidity curves in Fig. 3 shows at once that their mid-points do not exactly coincide. It is doubtful whether the point of equal solubility on either side of the isoelectric point can be considered as related to the position of the isoelectric point in the case of a highly polyvalent ion like that of protein, in the sense of the theory derived for ideal ampholytes. On the other hand, the nephelometrically determined turbidity value is determined by the number of particles of such a size as to be able to display the Tyndall effect, leaving aside the question of particle size which determines the color of the Tyndall light. At a given total protein concentration the number of particles will depend upon the solubility of the protein at the pH under consideration as well as upon the rate of aggregation to such particles. In the case of insulin we may consider the solubility as constant over practically the whole pH range under consideration. The turbidity values obtained at different levels of hydrogen ion activity at any given point of time may then be taken as an approximate measure of the rate of flocculation for that point of time. Further, the rate of flocculation is related to the electrokinetic potential of the protein at that pH. From this

point of view it is not surprising that the turbidity curve shows a fairly well developed maximum, whereas the minimum of solubility extends over a very broad range. No simple relationship between solubility and surface potential can yet be envisaged without assuming constant composition of the solid phase with varying pH. It is, however, justifiable to relate the position of the maximum flocculation point to the point of zero potential of the particle. We, therefore, consider the approximate value (pH 5.4) for the isoelectric point, as derived from the turbidity measurements, to be in essential agreement with the result of the electrophoretic measurement on adsorbed or amorphous insulin; and we are inclined to disregard as less significant the value of pH 5.55 to 5.60 derived from the solubility measurements.

#### SUMMARY

1. The isoelectric point of crystalline insulin, as determined from measurement in  $M/30$  acetate buffers of the electric mobility of insulin adsorbed on quartz or precipitated in amorphous form, is found to be between pH 5.3 and pH 5.35. Insulin crystals suspended in  $M/30$  acetate buffers are isoelectric at pH 5.0. The significance of this difference between the behavior of the surfaces of adsorbed and crystalline insulin is discussed.

2. Insulin is slightly soluble in  $M/30$  acetate buffers over a broad pH range extending from pH 4.8 to 6.5. The solubility in that range is constant, and of the magnitude of approximately 4 mg. per liter. The mid-point of the range of comparative insolubility lies at pH 5.55 to 5.60. This point is not considered to be a significant indication of the isoelectric point.

3. The maximum flocculation point of crystalline insulin determined nephelometrically in  $M/30$  acetate buffers lies at about pH 5.4, which is in essential agreement with the result of the electric mobility measurements.

*Addendum*—After the conclusion of this work a paper by Howitt and Prideaux (10), which is in essential agreement with the foregoing, appeared, giving measurements of the electrical mobility of gold particles coated with insulin, by the moving boundary method. The authors find the isoelectric point of insulin at pH 5.4, which is in excellent agreement with our results. Their figure was obtained by interpolation over about 2 pH units (between pH 4.2 and 6.8) in the region of insolubility. Though *amorphous* insulin of about the same activity as crystalline insulin was used in their

work, evidence was adduced showing that no fractionation of physiological activity had occurred during electrophoresis.

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## THE BIURET REACTION

### III. THE BIURET REACTION OF AMINO ACID AMIDES\*

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An investigation of the chemistry of the biuret reaction was begun in recent years by one of the authors (1, 2) to ascertain the nature and constitution of the colored products of the reaction, and the atoms in molecules showing the biuret reaction which take part in, and are essential for, the occurrence of the reaction. It is hoped that the data obtained from this study will furnish ultimately some clues regarding the molecular structure of the proteins, substances which so characteristically show the biuret reaction. The work just mentioned continues that of Schiff (3), who investigated qualitatively the behavior of a large number of compounds with the biuret reagents, alkali and cupric ion, and who was the first to isolate a product of the reaction, potassium copper biuret,  $K_2^+(Cu(biuret)_2)^- \cdot 4H_2O$ .

The investigations by Rising and her collaborators have demonstrated certain interesting facts regarding the formulas of the biuret reaction salts of di-acid amides and acid imides, and have led to the development of some useful theories concerned with the atoms which take part in the reaction, the structure of the salts formed, and the rôles of the biuret reagents. Some of these facts and theories have been discussed in previous papers (1, 2). The results of the study of the biuret reaction of amino acid amides, now to be described, offer further support for these earlier

\* The work here described forms part of the dissertation of Peter S. Yang, presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

The contents of this paper were reported at the national meeting of the American Chemical Society at Cincinnati, September, 1930.

theories, and indicate that the application of these theories is by no means limited to the biuret reaction of di-acid amides and acid imides. The experimental results augment the facts already ascertained regarding the chemical nature of the biuret reaction.

The investigation of the biuret reaction of  $\alpha$ -amino acid amides, compounds found by Schiff to show the reaction, was undertaken as a logical step leading to the study of the reaction of the proteins with the biuret reagents. The amides of *dl*-leucine, *l*-aspartic acid, *d*-alanine, and glycine were prepared (4), each amide was treated with the biuret reagents, and the colored "biuret salts" of the amides were isolated and analyzed. This study has brought to light certain likenesses, as well as some striking variations, in the behavior of amino acid amides with alkali and copper salts, as compared with the action of di-acid amides and acid imides under the same conditions. The biuret reaction of amino acid amides is less complex than that of the other types of compounds mentioned in that the reaction of the former occurs in the absence of alkali, producing internal salts having the empirical formula  $(\text{Cu}(\text{amino acid amide})_2) \cdot x\text{H}_2\text{O}$ . Products of this formula were formed when alkali was used and when it was not used, and no product of the biuret reaction of any amino acid amide studied contained alkali. In contrast to biuret salts of this type are those of di-acid amides, of empirical formula<sup>1</sup>  $\text{Me}_2^+(\text{Cu}(\text{di-acid amide})_2)^- \cdot x\text{H}_2\text{O}$ ,<sup>2</sup> and of acid imides, of formula<sup>3</sup>  $\text{Me}_2^+(\text{Cu}(\text{acid imide})_4)^- \cdot x\text{H}_2\text{O}$  (5), in which  $\text{Me}^+$  represents  $\text{Na}^+$  or  $\text{K}^+$ .

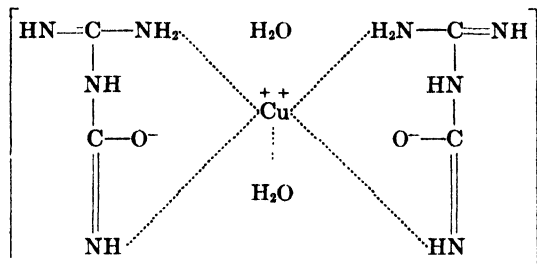
Many compounds which show the biuret reaction do so only in the presence of an excess of a strong base. The positive reactions of amino acid amides in the absence of alkali are not, however, the first instances to be noted of the occurrence of the biuret reaction without alkali. Monoiminobiuret,  $\text{NH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{C}-$

<sup>1</sup> Because of the instability of "biuret salts," their molecular weights have been difficult to determine. This has, however, been accomplished for potassium copper succinimide, and the data obtained prove that the molecular, as well as the empirical, formula of this salt is  $\text{K}_2^+(\text{Cu}(\text{succinimide})_4)^- \cdot 6\text{H}_2\text{O}$ . This work, by Mary M. Rising, L. B. Jefferies, and C. C. Li, will presently be published.

<sup>2</sup> Proved by Schiff to be correct for potassium copper biuret, and confirmed by Rising, Hicks, and Moerke for other di-acid amides.

<sup>3</sup> This formula has been confirmed by Rising and Johnson for the acid imide diethylbarbituric acid.

(:NH)NH<sub>2</sub>, and diiminobiuret, NH<sub>2</sub>·C(:NH)NH·C(:NH)NH<sub>2</sub>, react with copper salts in the absence of alkali to form deep red products of formula (Cu(iminobiuret)<sub>2</sub>)·xH<sub>2</sub>O, and are considered to show the biuret reaction (6). A constitution for the biuret salt of iminobiuret was first proposed by Rising and Johnson (1), which it is now desired to amend. The improved structure suggested follows.



Copper monoiminobiuret

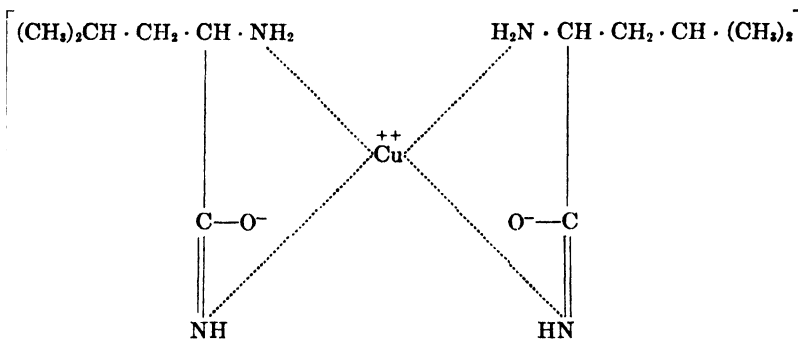
Here it will be seen that 2 molecules of an enol (acid) tautomer of biuret enter an ammonium-like complex with copper. The negative ion of the salt is within the complex and un-ionized, unlike the acid radical of copper ammonium sulfate, (Cu(NH<sub>3</sub>)<sub>4</sub>)<sup>++</sup> SO<sub>4</sub><sup>-</sup>. The structure of copper diiminobiuret may be represented in condensed form as (Cu<sup>++</sup>(HN:C(NH<sub>2</sub>)NH·C(:N<sup>-</sup>)NH<sub>2</sub>)<sub>2</sub>). (Coordination number of copper = 4.) In a general way this structure resembles that suggested for copper monoiminobiuret. It

will be noted that the amidine group, H<sub>2</sub>N·C:NH, of diiminobiuret is considered to function as does the amide group, H<sub>2</sub>N·C:O, of monoiminobiuret, but the latter must enolize before salt formation can occur. The amide group is probably more strongly acid than is the amidine group and would therefore react if present, but when no amide group occurs in a molecule showing the biuret reaction, as in diiminobiuret, the amidine group must perform function.<sup>4</sup> The central nitrogen atom of the biuret molecule is

<sup>4</sup> Amidines are known to form salts with bases in which the hydrogen atom of a —NH group is replaced by a metal ion, *e.g.* silver diphenylbenzamidine, C<sub>6</sub>H<sub>5</sub>·C(=NC<sub>6</sub>H<sub>5</sub>)N<sup>-</sup> (C<sub>6</sub>H<sub>5</sub>)Ag<sup>+</sup>

not believed to be involved in the biuret reaction; this conclusion has been drawn from the fact that malonamide,  $\text{NH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH}_2$ , containing a carbon atom instead of the central nitrogen atom of biuret, reacts with the biuret reagents exactly as does biuret itself.

There is a distinct parallel in the behavior of the iminobiurets and  $\alpha$ -amino acid amides with the biuret reagents, since the biuret salts of the latter compounds have the formula,  $(\text{Cu}(\text{amino acid amide})_2)$ , and alkali is not required for the biuret reaction of the amides. The first biuret salt of an amino acid amide prepared by us was copper leucinamide, obtained by treatment of *dl*-leucinamide with copper acetate with and without alkali. From the analytical data for the salt the empirical formula  $\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$  was calculated. The structure of copper leucinamide may be



Copper leucinamide

2 molecules of enol leucinamide enter a complex with copper. The negative radical of the salt is composed of the 2 enol ions, and is within the complex. It was anticipated that salts of this type should not conduct a current, since the ions are not free to move to the electrodes. This prediction was verified by experiment. Electrolysis of a neutral solution of copper leucinamide led to no conductance of the current and no migration of a colored ion to either electrode.

Electrolysis of a solution of copper leucinamide containing a small amount of alkali resulted in the migration of a colored ion to the anode. The effect of alkali upon copper leucinamide may

be to form a soluble compound, potassium copper leucinamide hydroxide, of formula  $K_2^+(Cu(OH)_2((CH_3)_2CH \cdot CH_2 \cdot CH(NH_2) \cdot CO^-(NH))_2)$ , somewhat as copper ammonium sulfate reacts with alkali to form copper ammonium hydroxide. In the former case the base must be un-ionized since the colored ion is negative, moving to the anode. That this substance was not formed instead of copper leucine when leucine was treated with alkali and copper acetate is in all probability due to the relative insolubility of copper leucinamide.

*d*-Alaninamide and glycinamide, like leucinamide, react with cupric ion to form colored salts of empirical formulas  $CuC_6H_{14}N_4O_2$  and  $CuC_4H_{12}N_4O_3$  respectively, and alkali is not required for these reactions. Structures analogous to that proposed for copper leucinamide have been formulated for these biuret salts. *l*-Asparagine reacts with copper acetate in the presence or absence of alkali to form a product containing no alkali ion. The empirical formula of copper asparagine is  $CuC_8H_{14}N_4O_6$ . This substance does not conduct a current in solution. The salt may be a true biuret reaction product of structure  $(Cu^{++}(CO_2-CH(N^+H_3)-CH_2 \cdot CO^-(NH))_2)$ . (Coordination number of copper = 2.) Or the compound may be a complex copper salt of the carboxylic acid asparagine, having the structure  $(Cu^{++}(CO_2-CH(NH_2)-CH_2 \cdot CO \cdot NH_2)_2)$ . (Coordination number of copper = 4.) In neither case are the ions free to conduct the current.

It is possible that the molecule of asparagine, which contains a free hydroxyl group, is neutralized by alkali when the amide is treated with cupric ion in the presence of a strong base and that a soluble salt, *e.g.* potassium copper asparagine, is formed. The compound copper asparagine may then be produced at the expense of the alkali copper salt, due to its greater insolubility.

The empirical formulas established for the biuret salts of the various types of compounds showing the biuret reaction, and studied so far, are set down here for ready inspection.

#### *Empirical Formulas*

Biuret salts of di-acid amides,	$Me_2^+$	(Cu (di-acid amide) $_2$ ) $^-$	$\cdot xH_2O$
“ “ “ acid imides,	$Me_2^+$	(Cu (acid imide) $_4$ ) $^-$	$\cdot xH_2O$
“ salt “ tetrapeptide (7),	$Me_2^+$	(Cu (peptide)) $^-$	$\cdot xH_2O$
“ salts “ amino acid amides,	(Cu (amino acid amide) $_2$ )		$\cdot xH_2O$
“ “ “ iminobiurets,	(Cu (iminobiuret) $_2$ )		$\cdot xH_2O$

It is undoubtedly significant that, in the cases so far studied, irrespective of the presence or absence of alkali in the biuret salt, the aggregate of molecules in the complex is just sufficient to make available 4 basic nitrogen atoms for taking part in the biuret reaction. These 4 nitrogen atoms may all be present in 1 molecule, as in the tetrapeptide to which reference has just been made, or in 2, or 4, molecules of the reacting substance, as indicated in the formulas.

The minimum number of acid hydrogen atoms involved in the biuret reaction seems to be 2, as in the case of the iminobiurets and amino acid amides. These compounds are strongly basic and no neutralization of acid groups by alkali is required to favor complex ion formation by the enhancing of the basic character of the nitrogen atoms. Biuret salts of such compounds therefore contain no alkali. When more weakly basic molecules showing the biuret reaction contain acid groups which require neutralization in order to increase the basic strength of the compound, as do the di-acid amides, acid imides, and peptides, 4 acid hydrogen atoms take part in the reaction, 2 of them reacting with alkali, and the biuret salts contain alkali. For the biuret reaction of such compounds salt formation is believed to be a fundamental preliminary to the occurrence of the reaction. The number of hydrogen atoms involved may very well depend upon the "acid-base balance" within the active molecules.

The development of the theory of the biuret reaction, based upon the results of further experimental work, will be continued.

#### EXPERIMENTAL

*Copper dl-Leucinamide*,  $CuC_{12}H_{26}N_4O_2$ —Copper *dl*-leucinamide was prepared as follows: (a) Without alkali. Solid *dl*-leucinamide (1.5 gm.), prepared by the method of Yang (4), was treated with a solution of copper acetate (2.2 gm. in 30 cc. of water). A pasty red substance formed and settled out completely upon the addition of a little water to the reaction solution. The compound was brought upon a filter, washed with water, alcohol, and ether successively, and dried over sulfuric acid. The yield was 1.1 gm. The analytical data follow (the copper content of this salt and of the others to be described later was determined by electrolysis.

Sodium was determined as sodium sulfate in solutions of the salts from which copper had been removed).

0.1159, 0.1105 gm. substance:	0.0226, 0.0218 gm. Cu
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. Cu 19.72
	Found. " 19.49, 19.72
0.0541, 0.0520 gm. substance:	0.0891, 0.0859 gm. $\text{CO}_2$ , 0.0409, 0.0388 gm. $\text{H}_2\text{O}$
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. C 44.75, H 8.13
	Found. " 44.91, 45.05, H 8.47, 8.34
0.1960, 0.1895 gm. substance:	HCl (factor 0.0978) 24.73, 24.50 cc.
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. N 17.40
	Found. " 17.27, 17.70

(b) With alkali. A solution of *dl*-leucinamide (0.5 gm. in 20 cc. of water) was treated with copper acetate (0.77 gm. in 10 cc. of water), the reaction mixture being stirred. The solution became reddish purple in color. A 40 per cent aqueous solution of potassium hydroxide (1 cc.) was then added drop by drop, whereupon a red precipitate formed. This was brought upon a filter, washed with very dilute ammonium hydroxide to remove copper hydroxide, then with water, alcohol, and ether. The salt was dried over sulfuric acid. The yield was 0.5 gm. The compound was analyzed and contained no potassium. The analytical data follow.

0.0636, 0.0507 gm. substance:	0.0125, 0.0100 gm. Cu
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. Cu 19.72
	Found. " 19.65, 19.72
0.0545, 0.0788 gm. substance:	0.0894, 0.1290 gm. $\text{CO}_2$ , 0.0397, 0.0574 gm. $\text{H}_2\text{O}$
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. C 44.75, H 8.13
	Found. " 44.73, 44.64, H 8.14, 8.15
0.0636, 0.0507 gm. substance:	HCl (factor 0.0978) 8.19, 6.63 cc.
$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ .	Calculated. N 17.40
	Found. " 17.60, 17.90

The analytical data indicate that the biuret salts of leucinamide obtained in the presence or absence of alkali are identical. This identity is shown further by the behavior of the two products. Each is a reddish amorphous substance which melts with decomposition at 248–250° (uncorrected) (8).<sup>5</sup> They are rather in-

<sup>5</sup> Bergell and Brugsch isolated a red copper derivative of leucinamide of melting point 222–223° (corrected). Analytical data: Cu 19.70, C 29.69, N 17.25, H 8.14.

soluble in water and in alcohol. The analytical data agree well with the empirical formula  $\text{CuC}_{12}\text{H}_{20}\text{N}_4\text{O}_2$ . The structure proposed for copper leucinamide is to be found in the introduction to this paper. This structure is supported by the behavior of the salt upon electrolysis. Its aqueous solution (50 mg. of the salt in 30 cc. of water) does not conduct a current.

*Copper l-Asparagine*,  $\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ —Copper *l*-asparagine was prepared by treatment of *l*-asparagine with copper acetate, and the product of the reaction was the same whether obtained in the presence or absence of alkali. When, for example, 2 gm. of powdered asparagine were suspended in 30 cc. of water and treated with concentrated aqueous potassium hydroxide until the amide dissolved and the solution became alkaline, the addition of saturated aqueous copper acetate dropwise to the alkaline solution resulted in the appearance of a purplish blue color. With continued addition of the copper acetate solution, precipitation of lavender copper asparagine occurred. Copper acetate solution was added until the precipitation of the biuret salt was complete; at this time the color of the solution had changed to blue. Some 20 minutes were allowed for complete precipitation; the salt was brought upon a filter, washed with a little 20 per cent potassium bicarbonate solution to remove copper hydroxide, then thoroughly with water, and was finally dried over sulfuric acid and analyzed. It contained no potassium.

0.1525, 0.1357 gm. substance: 0.0296, 0.0260 gm. Cu

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ . Calculated. Cu 19.52

Found. " 19.40, 19.16

0.1484, 0.2178 gm. substance: 0.1593, 0.2335 gm.  $\text{CO}_2$ , 0.0594, 0.0858 gm.  $\text{H}_2\text{O}$

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ . Calculated. C 29.48, H 4.30

Found. " 29.28, 29.24, H 4.47, 4.40

0.2573, 0.1923 gm. substance: HCl (factor 0.0987) 32.14, 24.41 cc.

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ . Calculated. N 17.20

Found. " 17.65, 17.40

Copper *l*-asparagine is a lavender amorphous substance which chars at 302–304° (uncorrected). The salt is rather insoluble in water and alcohol. The analytical data agree well with the empirical formula  $\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ . As stated in the introduction

the compound may be a true biuret reaction product, or it may be a complex copper salt of the carboxylic acid asparagine.

*Copper Glycinamide*,  $\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ —This salt was obtained (1) by treatment of glycinamide with copper acetate in the presence of alkali, and (2) by treatment of the amide with copper hydroxide in the absence of alkali. The products obtained by the two methods are identical and contain no alkali ion. When method (1) was used, glycinamide (1 gm.) was dissolved in 10 cc. of water and to this solution were added, with mechanical stirring, 3.9 cc. of 20 per cent aqueous sodium hydroxide. The reaction mixture was cooled meanwhile in an ice and salt bath. Then 10 cc. of an aqueous solution containing 1.8 gm. of anhydrous copper sulfate were added to the mixture drop by drop. The purple reaction solution was stirred for 2 hours, filtered, cooled in ice, and treated with 100 cc. of dry alcohol to precipitate potassium sulfate. When this salt was separated by filtration of the reaction mixture, 200 cc. of dry ether were added to the filtrate to precipitate copper glycinamide. The salt was brought upon a filter, washed with alcohol and ether, dried over phosphorus pentoxide, and analyzed. The yield was 0.8 gm.

0.0767, 0.0895 gm. substance: 0.0213, 0.0249 gm. Cu

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ . Calculated. Cu 27.91

Found. " 27.77, 27.82

0.1089, 0.0828 gm. substance: 0.0849, 0.0654 gm.  $\text{CO}_2$ , 0.0519, 0.0429 gm.  $\text{H}_2\text{O}$

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ . Calculated. C 21.08, H 5.31

Found. " 21.26, 21.54, H 5.33, 5.43

0.0790, 0.0902 gm. substance: HCl (factor 0.1128) 12.18, 13.92 cc.

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ . Calculated. N 24.61

Found. " 24.35, 24.37

Analysis of the copper glycine obtained by treatment of glycinamide with copper hydroxide without alkali gave data which agree with those just stated. All of the analytical results indicate an empirical formula  $\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ , or  $(\text{Cu}(\text{glycinamide})_2) \cdot \text{H}_2\text{O}$  for the salt. The compound obtained with and without alkali is a bluish pink amorphous substance having a decomposition point of  $205\text{--}207^\circ$  (uncorrected). It is readily soluble in water, and less so in alcohol. Aqueous solutions of the salt do not conduct a current.

*Copper d-Alaninamide*,  $\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ —This salt was obtained by treatment of *d*-alaninamide with (1) copper acetate in the presence of alkali and (2) copper hydroxide in the absence of alkali. Following the latter plan, a suspension of 0.55 gm. of freshly prepared copper hydroxide in 10 cc. of water was added slowly with mechanical stirring to 1 gm. of alaninamide dissolved in 10 cc. of water. The reaction mixture became deep purple in color. It was stirred for 2 hours, filtered, and treated first with 200 cc. of absolute alcohol, then with 800 cc. of dry ether, whereupon precipitation of orange-red copper alaninamide occurred. The salt was brought upon a filter, washed with dry alcohol and dry ether, dried over sulfuric acid, and analyzed. The yield was 0.50 gm.

0.0572, 0.0750 gm. substance: 0.0152, 0.0201 gm. Cu

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ . Calculated. Cu 26.74

Found. " 26.57, 26.80

0.0824, 0.0638 gm. substance: 0.0916, 0.0709 gm.  $\text{CO}_2$ , 0.0454, 0.0337 gm.  $\text{H}_2\text{O}$

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ . Calculated. C 30.29, H 5.93

Found. " 30.31, 30.30, H 6.13, 5.91

0.0608, 0.0587 gm. substance: HCl (factor 0.1128) 8.90, 8.70 cc.

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ . Calculated. N 23.57

Found. " 23.11, 23.40

The products obtained by methods (1) and (2) were identical, as shown by analytical data, color, and decomposition point (227–231° uncorrected). Results of analysis indicate that the empirical formula of the salt is  $\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ . The compound is very soluble in water, and only slightly so in alcohol. Its aqueous solution does not conduct a current.

#### SUMMARY

1. The "biuret salts" of glycineamide, *d*-alaninamide, *dl*-leucineamide, and asparagine have been isolated and studied.
2. The empirical formula of these biuret salts is  $(\text{Cu}(\text{amino acid amide})_2)$ .
3. The amino acid amides named show the biuret reaction in the absence of alkali.
4. Results of the work described support the theory that 4 basic nitrogen atoms are involved in a biuret reaction.

5. 2 acid hydrogen atoms take part in the biuret reaction of amino acid amides.

6. The number of hydrogen atoms involved in biuret reactions appears to be 2 or 4, depending upon the acid-base balance of the molecule showing the reaction.

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## EQUILIBRIA IN THE FORMOL TITRATION\*

By MILTON LEVY

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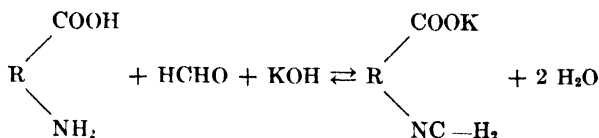
(Received for publication, October 28, 1932)

The theory of the titration of weak acids and bases has been systematized on the basis of titration constants characteristic of the acids and bases and a constant (such as  $K_w$ ) characteristic of the solvent and describing its behavior with strong alkalies. The usefulness of a knowledge of these characteristic constants is evident from the formidable literature which deals essentially with the behavior of acids and bases in water. The object of the present paper is to present data and an interpretation of them which may serve in an analogous way to systematize the behavior of certain of the monoamino acids in the formol titration. Through postulated equilibria the data are reduced to characteristic constants independent of the amount of formaldehyde in the solution. These constants, with a knowledge of the behavior of the solvent, should place the formol titration on a more satisfactory foundation than the present empirical one.

The formol titration depends on more or less marked shifts of certain parts of the titration curves of the amino acids in the presence of formaldehyde. The relationship of these shifts to stoichiometry was recognized by Schiff (1), who showed that some amino acids in formaldehyde neutralized an equivalent of alkali if phenolphthalein was used as an indicator. Sørensen (2) applied the possibilities of the formol titration as a quantitative method for the study of protein hydrolysis by acids, enzymes, etc. There is no need to emphasize the great usefulness of the method.

\* A preliminary report was given before the American Society of Biological Chemists at Philadelphia, April, 1932 (Levy, M., *J. Biol. Chem.*, **97**, p. xcii (1932)).

Sørensen (2) believed that the mixture of formaldehyde, amino acid, base, and water presented an equilibrium which he expressed in the equation.



The composition of the methylene compound was based on the substances which Schiff (3) had isolated from appropriate mixtures. Otherwise the equation and the existence of the equilibrium were based on the stoichiometry of the titration as it was affected by the formaldehyde concentration and the alkalinity of the end-point.

Harris (4) erroneously rejected this equation and interpretation because the titration of the amino acids in formaldehyde follows the Henderson-Hasselbalch equation ( $\text{pH} = \text{pK} + \log \alpha/1-\alpha$ ) at constant formaldehyde concentration. Application of the law of mass action to the Sørensen equilibrium leads to exactly this type of equation (as will be shown below) under the condition noted and Harris' rejection of the Sørensen interpretation is not valid. As far as the shape of the titration curve is concerned, Harris' experiments are consistent with the Sørensen equilibrium. Harris preferred to think of the system as dependent on a compound of the amino acid and formaldehyde having a dissociation constant differing from that of the original amino acid. While it is undoubtedly reasonable to assume that such compounds have definite hydron dissociation tendencies, the assumption is of little use, alone, in accounting for a variation of the observed titration constants with formaldehyde concentration. The additional assumption of an increase in the amount of the new acid with increasing formaldehyde concentration (Harris (5)) introduces into the equilibrium equations the acid corresponding to Sørensen's methyleneamino acid salt. This has proved to be unnecessary in the derivation of the equations which we use to describe the data presented in this paper. A sufficient assumption has been that formaldehyde combines with the amino acids only

to the extent that anions of the resultant compounds are formed.<sup>1</sup>

Derivation of equations including the presence of un-ionized formaldehyde compound is possible. Such equations show that when the amount of compound becomes appreciable the observed titration constants in formaldehyde no longer vary with the formaldehyde concentration. This is not observed in our data, so that we may conclude for the range of formaldehyde concentrations included, the amount of un-ionized formaldehyde amino acid compound is not significant.

Our success in fitting the variation of the titration constants with the assumption of the formation of two simple compounds of amino acid anion and formaldehyde leads to the rejection of the importance of the variation of the dielectric strength of solvent produced by the addition of the formaldehyde (Harris (7)).

Besides Schiff (3), a number of other workers have isolated substances from mixtures of amino acids or their salts and formaldehyde. (Among them are Franzen and Fellner (8), Krause (9), Bergmann and coworkers (10).) The compounds isolated do not seem to be particularly well defined. The possibility of the isolation of a particular compound depends on several factors besides its importance in an equilibrium between the various components of a system. The compounds are not of distinct value towards a formulation of the system involved in the formol titration, except that they support the existence of compounds formed from alkali, amino acids, and formaldehyde containing from 1 to 3 molecules of formaldehyde or its residue for each molecule of amino acid.

*Zipper Ionen and Formol Titration*—Harris (11) (1930) uses the behavior of the amino acids in formaldehyde as a proof of the *Zipper Ion* structure for amino acids. The logic involved in this proof may be stated without details in the form of the following syllogism. (1) The titration constants of amines are shifted towards diminished basicity in formaldehyde solutions. (2) The

<sup>1</sup> Svehla (6) has shown by freezing point measurements that compounds are formed between amino acids and formaldehyde without the addition of alkali. These compounds are apparently not important in determining the behavior of the systems when alkali is added.

NaOH titration constants only of the amino acids are diminished by formaldehyde. (3) Therefore the titrations of amino acids with NaOH are reactions of the amino groups of the amino acids.

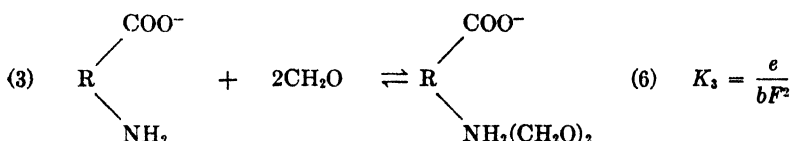
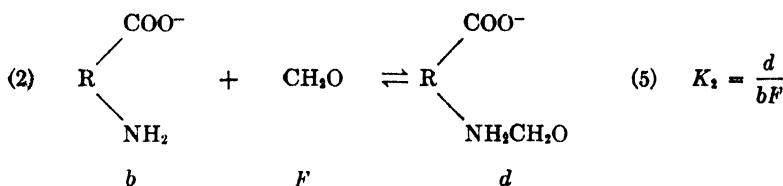
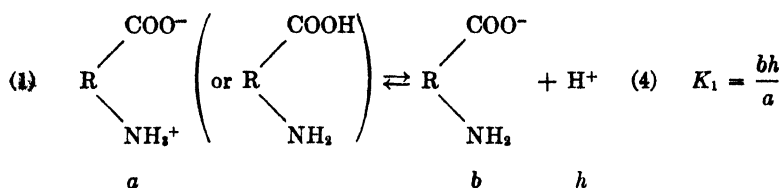
The acceptability of this syllogism would seem to depend on the interpretation of the term "titration constant." If one accepts, as Harris does, the existence of formaldehyde-amino acid compounds whose dissociation constants may be measured in the formol titration then one may accept this as a proof of the thesis that the NaOH titration constant represents a reaction of the amino group and not of the carboxyl group of the amino acid. But if the reaction is interpreted as Sørensen interprets it and as it is developed in the present paper then the titration constant becomes a composite which may be made to depend on the affinity of the amino acid anion (independently of its mode of formation) for formaldehyde and the hydrion dissociation constant of the amino acid as measured by the ordinary NaOH titration. It is therefore of no material consequence with what group the hydrion was previously associated and Harris' argument reduces to an analogy with the validity of Bjerrum's (12) original interpretation of the ampholyte constants.

The concept of *Zwitter Ion* is still satisfying but the proof of its reality must depend on a method the results of which can be interpreted in only one way. It may be pointed out that for a systematization of the knowledge of the thermodynamic behavior of acids and bases with respect to the hydrogen electrode the Bronsted (13) nomenclature allows completeness without involving questions as to the structure of ampholytes and without requiring that certain constants be called acid constants and others basic constants in the old sense.

### *Theoretical*

During our studies of the formol titration we have developed the following theory of the titration which numerically accounts for the data on the basis of experimentally determined constants.

The following equilibria are postulated to determine the behavior of the system.



Equations 4 to 6 are the corresponding equilibrium equations derived from the law of mass action.  $a$ ,  $b$ ,  $h$ ,  $d$ , and  $F$  are concentrations (used in place of activities) of the various ions and molecules below which they appear in the chemical equations.  $K_1$  is the hydrogen ion dissociation constant of the amino acid.  $K_2$  and  $K_3$  are association constants between the amino acid anion and 1 and 2 molecules of formaldehyde respectively.

If  $\alpha$  be defined as the fraction of the total amino acid present as one form or other of the anions, and complete dissociation of salts is assumed, Equation 7 results.

$$(7) \quad \frac{\alpha}{1 - \alpha} = \frac{b + d + e}{a}$$

From Equation 7 and the equilibrium equations there may be derived Equation 8.

$$(8) \quad \text{pH} = -\log (K_1 + K_1K_2F + K_1K_3F^2) + \log \alpha / 1 - \alpha$$

The application of this equation to the formol titration requires either that  $F$  be replaced by the exact relationship

$$(9) \quad F = C - d - 2e$$

in which  $C$  is the formaldehyde added to the mixture or that an approximation be introduced. The use of the exact relationship leads to a complicated relationship and an approximation is readily available. If the total amino acid concentration is small compared to the formaldehyde concentration then  $d$  and  $e$  will be small compared to  $C$  and under these conditions it is permissible to neglect the amount of formaldehyde combined and to substitute for  $F$  in Equation 9 the total added formaldehyde.

Therefore at a constant and sufficiently high formaldehyde concentration the first term on the right of Equation 8 becomes constant and equal to the pH at the mid-point of titration of the amino acid. This titration constant may be designated  $pG_f$  and defined by Equation 10.

$$(10) \quad pG_f = -\log (K_1 + K_1 K_2 F + K_1 K_3 F^2)$$

The similarity of form between Equation 8 and the Henderson-Hasselbalch equation ( $pH = pK + \log \alpha/1-\alpha$ ) may now be readily recognized.

Equation 10 may be transformed into a suitable form for graphic determination of  $K_2$  and  $K_3$  by taking antilogarithms and rearranging terms to obtain Equation 11.

$$(11) \quad \left( \frac{G_f}{K_1} + 1 \right) \frac{1}{F} = K_2 + K_3 F = M$$

If the function  $M$  defined by Equation 11 be plotted against  $F$ , then a straight line should result, the intercept of which is  $K_2$  and the slope  $K_3$ .

At the higher formaldehyde concentration  $K_3 F^2$  becomes large in comparison to  $K_2 F$ , and as an approximation, the validity of which will vary from one amino acid to another, the terms  $K_1$  and  $K_1 K_2 F$  may be neglected in Equation 10 and Equation 12 results.

$$(12) \quad pG_f = -\log K_1 K_3 - 2 \log F$$

The constant  $-\log K_1 K_3$  may be determined from a plot of  $\log F$  against  $pG_f$ . Such curves reach or approach a slope of  $-2$  as is expected from Equation 12 and the intercept extrapolated on this slope is the value of  $-\log K_1 K_3$ .

The addition of Equations 1 and 2 gives a modern equivalent of the Sørensen equilibrium. It is only necessary to place  $K_3 = 0$  in Equation 8 to obtain the equations which would be applicable in this case. It is therefore seen that the Sørensen equilibrium leads to the form of the Henderson-Hasselbalch equation contrary to Harris' (4) statement that it does not.

#### EXPERIMENTAL

In his earlier work Harris used an indicator method for the determination of pH but later showed that the hydrogen electrode could be used in formaldehyde solutions. The latter method has been used exclusively in obtaining the data in the present paper. We have also avoided the use of commercial formalin of qualitatively and quantitatively unknown composition.

The cell used consisted of a saturated KCl calomel half cell, an agar KCl bridge, and the solution containing a platinized platinum electrode; the solution and electrode were in a closed vessel through which hydrogen passed. Temperature was controlled by means of an air thermostat at 30° and all solutions were brought to this temperature before use. The titrating reagent was added from a burette graduated at 0.01 ml. intervals. The potentials were measured by the use of the usual potentiometer and galvanometer.

While it is quite easy to detect the odor of formaldehyde over quite dilute solutions of formaldehyde, no evidence that significant quantities of formaldehyde were carried off in the hydrogen stream was obtained. No regular drifts of potential occurred on changing the rate of bubbling and in some cases the hydrogen was first passed through a formaldehyde solution of the same concentration as that in the cell but this precaution seemed unnecessary within the limits of variation desired.

The amino acids were obtained from commercial sources or prepared in the laboratory, except for leucine which was kindly supplied by Dr. H. B. Vickery.

The formaldehyde was prepared by the dry distillation of para-formaldehyde. The gas was led through wide tubes into distilled water. The solution so obtained was filtered and the formaldehyde content determined by oxidation with alkaline iodine (Rommijn (14)). The stock solution of formaldehyde approximated 10 M and after 10-fold dilution had a pH between 5 and 6. This was

taken to indicate that formic acid was not present in significant quantities.

Formaldehyde neutralizes a certain amount of base in alkaline solutions. The correction or blank on the formaldehyde depends on the formaldehyde concentration. It was found unnecessary to correct any of the  $pG_f$ ,  $\log F$  curves for this but in the titration with base at constant formaldehyde concentration the end-point is obscured in certain cases unless a formaldehyde blank is run. Since no data are given in this paper involving these blanks they are not presented here.

### Results

*Form of Titration Curve*—The statements of Harris (4) that the form of the titration curve in formaldehyde is like that in water has been fully confirmed for a number of the amino acids at low amino acid concentrations and at all formaldehyde concentrations dealt with. It is not felt necessary to present the actual data, as in the main they are simply a duplication of Harris' results. These results are in conformity with Equation 8.

*Variation of  $pG_f$* —In order to follow the variation of the titration constant in formaldehyde the following procedure was carried through. The amino acids were brought to the mid-points of the alkaline branches of their titration curves by the addition of the appropriate quantity of NaOH. The solution so prepared was placed in the titration vessel and the pH measured. This could be compared with the known titration constant of the amino acid. A formaldehyde solution of known concentration was added from the burette and the pH measured after each addition. The concentration of formaldehyde in the solution was calculated from the concentration in the titrating fluid and the volume added on the assumption that the volumes of the original solution and titrating solution were additive. Details were so arranged that the concentration of amino acid and its salt remained approximately constant (within 10 per cent) throughout the titration. The pH measured at each formaldehyde concentration is equal to  $pG_f$ . In Fig. 1 the values of  $pG_f$  are plotted against the logarithms of the formaldehyde concentrations and a smooth curve drawn through the points.

An inspection reveals that these curves reach or approach a slope of 2 as the formaldehyde concentration increases (except proline in which the curve reaches a slope of 1). In the plot these portions of the curves have been extrapolated to  $\log F = 0$  and the constants ( $-\log K_1K_3$ ) of Equation 12 have been read. In the case of proline the intercept is equal to  $-\log K_1K_2$  of Equa-

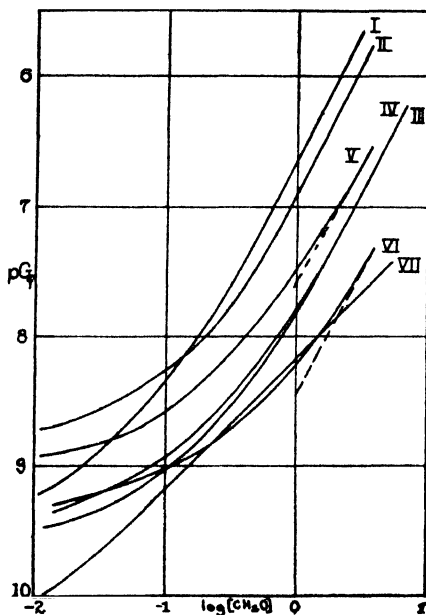


FIG. 1. The variation of titration constant ( $pG_T$ ) with the logarithm of the formaldehyde concentration. Curve I represents glycine (0.005 M); Curve II,  $\alpha$ -amino- $\beta$ -phenylacetic acid (0.005 M); Curve III, leucine (0.01 M); Curve IV, glutamic acid (0.005 M); Curve V, phenylalanine (0.005 M); Curve VI, tyrosine (0.002 M); Curve VII, proline (0.01 M). The dash lines represent extrapolations.  $[CH_2O]$  is in mols per liter.

tion 8 and  $K_3$  is equal to 0. The values of these constants are given in Table I along with  $pK_1$  and the pH of 99 per cent neutralization in 10 per cent formaldehyde calculated from the constants.

The constant,  $-\log K_1K_3$ , is a practically useful constant since from it one may determine the titration constant at a given formaldehyde concentration. The equation to which it applies is valid for the amino acids studied from about 2 M (6 per cent) for-

maldehyde upward and therefore is applicable in the formol titration as usually carried out (5 to 20 per cent formaldehyde).

The proof of the validity of the entire theory developed in the theoretical section depends on the applicability of Equation 10 to the data. In Fig. 2 the function  $M$  of Equation 11 is plotted against the formaldehyde concentration  $F$ . The points fall reasonably well on straight lines and from these lines the slopes ( $K_3$ ) and intercepts ( $K_2$ ) may be read. Many determined points have been omitted from the plot and in order to get the curves on one graph some portions of the curves have been omitted.

TABLE I

*Formol Titration Constants Based on the Relationship,  $pG_f = -\log K_1K_3 - 2 \log (CH_2O)$ , at 30°*

Amino acid	$-\log K_1K_3$ (30°)	$pK_1$ (30°)	pH of end-point (99 per cent neutralized) in 10 per cent $CH_2O$
Glycine	6 65	9 6	7 6
$\beta$ -Phenyl- $\alpha$ -aminoacetic acid	6 90	8 84	7 9
Phenylalanine	7 57	8 99	8 6
Glutamic acid	7 87	9 32	8 9
Leucine	7 87	9 50	8 9
Tyrosine	8 45	9 07	9 5
$pG_f = -\log K_1K_2 - \log (CH_2O)$			
Proline	8 25	10 30	9 7

We may conclude from the fit of the graph that the equilibria postulated in the theoretical section are justified in that they lead to a numerical equation which describes the data. A reservation is necessary in regard to glycine. There is evidence in this case that the formaldehyde compound is polymerized to a marked extent and the values of the constants are dependent to some extent on the actual glycine concentration. We have in mind a further development of the theory to account for the behavior of glycine. In all the data presented in this section the amino acids have been at a concentration of 0.01 or 0.005 M and except for glycine no significant variation with amino acid concentration was observed.

In Table II the values of  $K_2$  and  $K_3$  for a number of amino acids are given as determined from Fig. 2. The presence of three con-

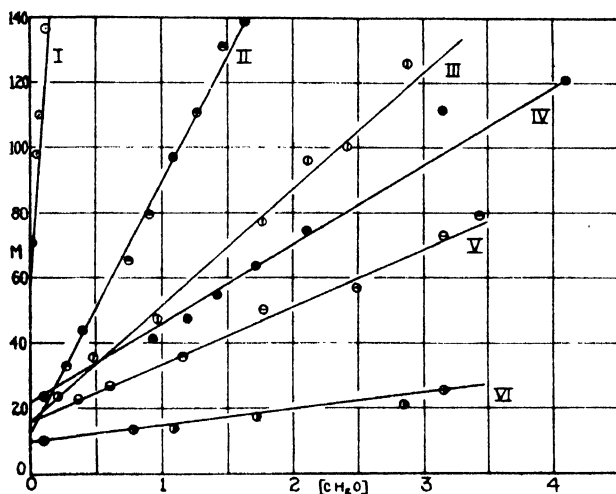


FIG. 2. Test of Equation 11, plot of  $M = \left(\frac{G_f}{K_1} - 1\right) \frac{1}{F}$  against  $F$ . Curve I represents glycine (0.005 M); Curve II,  $\alpha$ -amino- $\beta$ -phenylacetic acid (0.005 M); Curve III, leucine (0.01 M); Curve IV, glutamic acid (0.005 M); Curve V, phenylalanine (0.005 M); Curve VI, tyrosine (0.002 M).  $[CH_2O]_f = F$  is in mols per liter.

TABLE II  
Formol Titration Constants Referring to Equation 8

Amino acid	$K_2$	$K_3$
Leucine.....	16	35
$\beta$ -Phenyl- $\alpha$ -aminoacetic acid.....	13	77
Glutamic acid.....	22	24
Phenylalanine.....	16	23
Tyrosine.....	10	5
Glycine.....	60	290 (0.01 M only)

stants in Equation 8 is somewhat deceptive since one of them ( $K_1$ ) is independent of the measurements described.

For proline the variation of  $pG_f$  is determined by the Sørensen equilibrium alone and the behavior of the system present in the

formol titration of proline can be accounted for on the basis of the hydrion dissociation constant of proline and an association constant of the proline anion and formaldehyde. This constant is  $K_2$  in the scheme derived above. This difference between proline and the true amino acids is no doubt significant for the structure of the formaldehyde compounds.

#### DISCUSSION

The significance of the results for the practical formol titration will not be completely evident until a study has been made of the behavior of the formaldehyde solution with alkali alone. This behavior will determine the nature of the second factor involved in the determination of the end-point of the formol titration. Thus while it might appear from the data presented that the titration in formaldehyde would be most accurate at the highest possible formaldehyde concentration (lowest  $pG_f$ ) this conclusion is erroneous because of the nature of the blank titration. The most favorable formaldehyde concentration for the formol titration will undoubtedly prove to be a compromise between a low  $pG_f$ , favored by high formaldehyde concentration, and a low blank favored by low formaldehyde concentration.

An important practical point is that the various amino acids have different characteristic constants for their equilibria with formaldehyde. This may become of importance in the titration of mixtures of amino acids such as appear in biochemical work. Ideally we would wish to titrate all of the amino acids in such a mixture but we cannot doubt that the best solution will again be a compromise.

The basic amino acids, lysine, histidine, and arginine, present complications due to the number of groups involved and their effect on one another. Data on their titrations in formaldehyde have been accumulated to some extent but presentation must await completion of the work. These amino acids will be particularly interesting because of their importance in determining the titration curves of proteins.

#### SUMMARY

A theory and equations describing the behavior of amino acids in the formol titration have been evolved based on the following postulates.

1. Only amino acid anions react significantly with formaldehyde.
2. Two types of new anions are formed, one involving 1 molecule of formaldehyde per amino acid anion and the other 2.

The equations developed have been applied to data and found to describe the system adequately.

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## **SIMULTANEOUS STUDY OF CONSTITUENTS OF URINE AND PERSPIRATION**

BY H. H. MOSHER

*(From the Climax Rubber Company, New York)*

*(Received for publication, November 16, 1932)*

Although much attention has been directed to the study of the chemical components of human urine and blood with the aim of establishing relationships between the presence of certain bodies and variations in the percentages of the normal constituents with pathological disorders, few similar investigations have been conducted with perspiration. This is indeed surprising when it is considered that many dermatologists ascribe certain skin diseases to unknown chemical substances or to an abnormal balance of the usual constituents in the sweat.

Before any useful investigations on the influence of perspiration on the etiology of skin disorders can be scientifically instigated, more exact information as to the normal components of sweat is required and this is particularly true of the nitrogen fraction. The data on this subject are meager and much of the information is inexact and has been secured by methods hardly calculated to furnish reliable results.

As far as we are aware no complete analysis of the normal perspiration has been recorded. Individual components have been detected and in some cases their maximum and minimum limits established under certain specified conditions, but save for a few exceptions in which total solids, chlorides, non-protein nitrogen, and urea were simultaneously determined on the same sweat sample, almost all studies have been confined to one or two components. This is particularly true of the nitrogen fraction.

We were particularly interested in the non-protein nitrogenous fraction, the reaction of normal perspiration, and in those bodies producing the characteristic perspiratory odors. A correlation between the sweat and urine constituents during the period of test was also desired.

## 782     Constituents of Urine and Perspiration

This has been made the subject of a similar investigation to the best of our knowledge only by Talbert and his coworkers. In a study extending over a number of years these investigators have found that sweat contains urea (1), uric acid (2), amino acids (3), ammonia (4), lactic acid (2), and sugar (5). The quantities of each of these components varied widely.

They found that perspiration normally contains between 0.24 and 1.12 mg. of urea per cc. Such values would account for from but 1.5 to 7 per cent of the urea eliminated through the skin daily, assuming a sweat secretion of 600 cc., and hardly are in accordance with Cramer's (6) estimate of 12 per cent. It is probable however that Talbert's figures are much lower than the normal values of the urea content in sweat. Riggs (7) has shown that as the flow of perspiration is stimulated and becomes more copious the organic matter in the fluid is reduced and is inversely proportional to the amount of sweat exuded through the skin. Talbert's samples were obtained by artificially inducing a flow from the sweat glands by pilocarpine and heat. Marshall (8), Barney (9), and others verified Talbert's value with samples obtained in a like or similar manner. In certain pathological disorders such as cholera and uremia the sweat glands may assume some of the functions of the kidneys and excrete urea of such quality that the entire skin surface is covered with very fine crystals (10).

Uric acid has been detected in sweat by a few experimenters but the amounts detected varied widely. Talbert and coworkers (2) and Voit (11) found very minute quantities in normal perspiration. Adler (12), on the other hand, detected very considerable quantities which in some cases reached the high figure of 30 mg. per 100 cc. According to Barney (9), uric acid was absent from the perspiration of the subjects that he studied.

The very accurate and sensitive amino acid determination methods of Folin (13) and of Van Slyke (14) have enabled experimenters to obtain very consistent results of the amino acid nitrogen content of perspiration. Talbert and coworkers (3), Embden and Tachau (15), and Neuberg (16) all report values ranging between 1.6 and 6.4 mg. per 100 cc.

Ammonia is an easily demonstrable component of perspiration and has been detected by all experimenters who sought its pres-

ence. Most of the nitrogenous bodies in sweat, however, are very easily fermented by bacterial and fungous agencies yielding ammonia; and, as most investigators of the composition of sweat have taken no care in obtaining or maintaining antiseptic conditions, it is probable that the ammonia contents reported are too high. Talbert and coworkers (4) obtained values of from 0.04 to 0.2 mg. per cc. and such values are about in accordance with the results of other experimenters.

The presence of creatinine and creatine in perspiration is still a controversial matter. Several investigators including Cramer (6), Capranica (17), and Schumann (18) have identified these end-products of protein metabolism in sweat samples and Schumann believes them to be normal constituents of human sweat. The amounts found were in no case large. Schumann believes that while creatinine is ordinarily present in amounts ranging from 3.5 to 5 mg. per cc. the creatine content is hardly greater than mere traces. Talbert and his coworkers (5) have been unable to secure any indication of either of these products, however.

Other non-protein nitrogenous substances have been reported as having been isolated from perspiration but the nature of the compounds described justifies the belief that they are chance contaminants and not ordinary normal or abnormal sweat constituents. It is probable that minute traces of the purines and other nitrogenous bodies are present in sweat and that the character of these products is as complicated and diversified as in urine itself.

The chemical reaction of perspiration has been made the subject of a considerable number of studies. Most experimenters have found human sweat slightly acid and some relationship between this reaction and dermatological disorders has been reported. However, the connection is by no means conclusive and might as readily be attributed to other causes. Owing to inaccuracies in measuring acidity, much of the early work is almost valueless, and even the results of later investigators show such variations as to render the conclusion that the reaction of sweat is a very variable factor inevitable. However, it has been pointed out first by Schiefferdecker (19) and later by Fishberg and Bierman (20) that sweat may be secreted from two different types of glands, which has been called exocrine and apocrine sweat. These fluids vary considerably in composition and reaction and their flow is

stimulated by different incitants. It is probable that the variations in the relative degree of acidity reported by different experimenters can be partially explained by different ratios of the two types of sweat in the samples. According to different investigators, the pH of perspiration may vary from 3.4 to 8.4.

Sugar has been identified in perspiration by Talbert and his coworkers (5), Usher and Rabinowitch (21), Sutton and Sutton (22), Cornbleet and Montgomery (23), and others and appears to be a normal constituent of this fluid. It is largely due to this substance that sweat acts as an excellent culture medium for bacteria, fungi, and other microorganisms. This fact may prove of interest in bacterial skin infections. The percentage of sugar in the perspiration is generally low, ranging from 2.8 to 50 mg. per 100 cc. of sweat. Diabetic patients, as might be expected, have a much higher percentage of sugar in their perspiration than has the normal individual (24) and are more prone, as a natural corollary, to fungous skin infections.

Lactic acid and lactates are present in all sweat and the amount appears to vary with the amount of exercise taken by the subject immediately before collecting the samples. Krestownikoff (25) found that the average individual's perspiration contained about 15 mg. per 100 cc. but increased rapidly during participation in sport and might reach values of over 100 mg. Koriakina and Krestownikoff (26) found 1765 mg. of lactic acid per 100 cc. in the sweat of Marathon runners. Talbert, on the other hand, found that the lactic acid content of sweat in the average person was considerably higher than Krestownikoff's figure, ranging from 71 to 160 mg. per 100 cc.

The mineral bodies consist chiefly of sodium chloride, alkali sulfate, phosphates, and unknown complexes of calcium and magnesium (20, 27). The relative amounts of these in perspiration are said to differ materially from those in urine (28). Most of the sulfur is present not as the inorganic sulfate but as ethereal sulfuric acid esters. The chloride content varies enormously with the subject and seems to bear some relation to diet and individual idiosyncrasies. Talbert and Haugen (29) found the limits to range in the subjects that he studied between 420 and 660 mg. per 100 cc. These values roughly approximate those secured by other observers (30, 31), though results as high as 2 per cent have been recorded.

A study of the composite information on perspiratory components indicates that perhaps a very close physiological relationship exists between urine and sweat. Both fluids contain many of the same chemical substances and have a similar acid reaction, and it might be deduced would have a similar effect upon the skin.

However, the chemical data on sweat are too incomplete to permit of accurate postulations. We have been unable to discover any record of a complete chemical analysis of the non-protein nitrogenous end-products of protein metabolism in sweat samples, or data which would furnish a daily average of any single component. Samples collected at a specified time record only the composition of the perspiration exuded at that period and may only roughly indicate the average. It is well known that urine varies greatly throughout the day and a true average is only obtainable upon the collection of all voided during a 24 hour period. The same condition probably exists with sweat.

All of the tests described in the literature on perspiration composition were made with specimens collected under aseptic conditions. Sweat is an easily fermentable fluid, being readily changed by bacterial and fungous agencies. While such changes may not produce any great variations with more concentrated solutions, the minute quantities of such nitrogenous substances as uric acid and creatinine occurring in sweat may be largely converted into other bodies during the time required to collect the samples.

Most experimenters have collected their samples by placing the subject in rubber sheets or enclosing a portion of the subject's anatomy with some type of rubber jacket. No indication exists from their articles on the subject that any particular precautions were exercised to prevent vitiation of the samples by rubber-extracted material. Almost all rubber sheets and articles of rubber construction contain small amounts of sweat-soluble matter which may be acid or alkaline depending upon whether the crude rubber from which the rubber has been derived was acidic or basic, and upon the nature of those auxiliaries used in the manufacture of rubber sheet. These rubber extractives might profoundly influence the analysis of the materials collected unless the rubber had been specially treated to remove soluble matter.

In our study of the urinary and perspiratory products three

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males in excellent physical and normal mental condition were selected as the subjects for investigation. All maintained their normal routine during the period of test. No dietary changes were prescribed and no effort made to control their moisture intakes. The investigation was of but 2 days duration.

All urine voided by each subject was collected separately and furnished two 24 hour samples. The collection receptacles contained a small amount of c.p. chloroform to prevent decomposition.

Samples of perspiration were collected from each subject three times daily, at 9 a.m. and at 3 and 11 p.m. It was felt that the mean of the three would furnish a value approximating rather closely the daily average of the sweat composition of each subject. Immediately after the collection of the morning and afternoon samples, they were submitted to chemical analysis. The night sample was preserved with a few additional drops of chloroform and refrigerated until the next morning. About 200 cc. of perspiration were collected from each subject at each sweating operation.

The bodies of the patients, prior to the collection of the samples, were very thoroughly washed with warm water in order to remove soluble matter and loosely adherent dirt and cutaneous bodies. Possible urinary contaminations of the sweat samples were prevented by encasing the excretory organs of the subjects in tight fitting rubber caps prior to the collection of the perspiration.

The subjects were then introduced into a sweat chamber maintained between 40–50° and placed on special beds, the mattresses of which were covered with tautly stretched purified rubber sheets which gently sloped from the sides to the center. At the low points glass tubings pierced the mattresses and were attached to the sheets, which had small holes at these points. The lower ends of the glass tubes communicated with receptacles for sweat collection, containing a few drops of c.p. chloroform. The patients were covered with purified rubber sheets and finally with blankets. As sweat was exuded from the bodies of the patients, it gradually flowed to the low point of the lower sheet and into the receptacle below. In this manner, bacterial and fungous decomposition of the non-protein nitrogenous matter of the perspiration was largely arrested.

It was found that from  $\frac{1}{2}$  hour to 50 minutes were required to

secure the 250 cc. of sweat required for the determination. No pilocarpine or other sweat-stimulating agents were employed to induce more copious sweating.

The rubber sheets were prepared from pure crêpe rubber and were washed until they were found free from all sweat-soluble extractives by determination.

Both the urine and sweat were analyzed for total solids, chlorides, sulfates, organic matter, non-protein nitrogen, sugar, lactic acid, and the non-protein nitrogen fractions urea, uric acid, ammonia, amino acid nitrogen, creatinine, and creatine.

The total solids were determined by evaporating 5 cc. samples to constant weight at 80°. Organic matter was found in accordance with the conventional technique on the evaporated material employed from the total solid determination.

Chlorides were found by precipitating the chlorine in the ashed samples with standard silver nitrate solutions and titrating the excess with standard ammonium sulfocyanate, with ferric ammonium sulfate as an indicator.

Sulfates were determined by the conventional barium chloride procedure.

The customary procedures of Folin and coworkers (13, 32) were utilized in establishing the contents of the sugar and non-protein nitrogenous components of the sweat. Minor modifications were adopted in certain procedures but these consisted chiefly in avoiding dilution of the sweat, which is a very dilute solution.

The lactic acid content of the sweat was determined by the gasometric method of Avery and Hastings (33). Unfortunately, the determination of this component in urine did not prove satisfactory and the results are not included in this paper. Whether our inability to obtain accurate lactic acid values in urine was due to faulty technique, undetermined impurities in the urine, or some other cause was not investigated.

The results of the analysis are recorded in Table I. All results on sweat are the mean from the three samples from each subject collected daily. We were unable to establish any quantitative correlation between the urinary and perspiratory components. Accordingly, the urinary analyses are not included in Table I, as they show nothing novel.

The results on the amounts of the individual components of the

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human sweat samples are about in accordance with values reported on individual determinations by other observers. We did, however, secure a relatively higher percentage of the non-protein nitrogen fraction as urea and a correspondingly lower amount of ammonia. We are inclined to attribute this to efforts made to prevent alterations in the sweat by bacterial and fungous agencies during the period of time required for collecting the samples. Both the uric acid and creatinine fractions of the sweat were quite

TABLE I  
*Composition of Sweat and Urine*

Subject A, male, 28 years of age. Subject B, male, 39 years. Subject C, male, 36 years.

All results are computed as mg. per 100 cc. of fluid.

Day.....	Sweat						Urine, average of all on both days
	Subject A		Subject B		Subject C		
	1	2	1	2	1	2	
Amount collected, cc .....	583	540	470	440	534	498	6870
pH.....	5.42	5.65	5.02	5.18	5.71	5.47	5.91
Total solids.....	1380	1305	1280	1174	1597	1207	4365
Mineral matter.....	1027	985	991	821	1170	890	2300
Chlorides (Cl).....	582	576	508	395	602	485	938
Sulfates (SO <sub>4</sub> ).....	12	8	7	5	4	17	240
Non-protein N.....	75	66	81	77	108	86	1006
Urea N.....	50	40	56	51	81	62	814
Ammonia N.....	11	8	15	8	11	14	34
Uric acid N.....	0.4	0.3	0.2	0.2	0.5	0.3	43
Creatinine “.....	0.8	0.7	0.2	0.5	0.7	0.5	58
Creatine N.....	None	Trace	None	None	Trace	Trace	6
Amino acid N.....	3.1	2.4	3.3	2.9	3.7	2.9	13.7
Sugar.....	21	6	8	18	22	16	71
Lactic acid.....	94	107	34	58	78	71	Present

low and present in hardly more than traces. Our results regarding the uric acid of perspiration confirms the work of Talbert and his coworkers and the experiments of Voit. Qualitatively our results confirm those of Schumann regarding the presence of creatinine in sweat, though our values were much lower than any he records.

### SUMMARY

Qualitatively perspiration was found very similar to urine in composition, though the relative amounts of the various com-

ponents in the two solutions varied considerably. Urine is a much more concentrated solution, containing from 3 to 5 times the amount of total solids and from 5 to 9 times the amount of organic matter.

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